

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

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ABSTRACT

Enhanced stress response has been suggested to promote longevity in many species. Calorie restriction (CR) and conserved nutrient-sensing TOR and 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 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 general stress response transcription factors Msn2, Msn4, and Rim15. The *bmh1* Δ mutant also displays decreased ROS level and increased HSE (heat shock elements)-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.

INTRODUCTION

Recent studies in genetically tractable model systems including yeasts, worms, flies and mice demonstrated that longevity could be modulated by single gene mutations (DILOVA *et al.* 2007; KENYON 2001; TISSENBAUM and GUARENTE 2002). In addition to genetic interventions, calorie restriction (CR) has also been shown to extend life span in many species (ROTH *et al.* 2001; WEINDRUCH and WALFORD 1998), 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 an individual yeast cell undergoes before senescence. CLS measures the length of time cells remain viable at a non-dividing state. Yeast cells enter non-dividing stationary phase when nutrients are exhausted. This quiescent state has been suggested to resemble the G₀ 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 (EASLON *et al.* 2007; LIN *et al.* 2000; 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-cAMP/PKA (cyclic-AMP activated protein kinase A) pathway,

which regulates cell growth and stress response, extends life span (FABRIZIO *et al.* 2001; LIN *et al.* 2000). Deleting the nutrient responsive kinases Sch9 (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 (EASLON *et al.* 2007; LAMMING *et al.* 2005; LIN *et al.* 2000). Interestingly, Sir2 appeared to be dispensable for CLS (FABRIZIO *et al.* 2005; SMITH *et al.* 2007) and RLS (EASLON *et al.* 2007; JIANG *et al.* 2002; KAEBERLEIN *et al.* 2004) 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.

Enhanced stress response has been shown to play important roles in extending longevity. Increased stress response may protect cells from age-dependent accumulation of damages caused by reactive oxygen species (ROS) generated as metabolic byproducts. In yeast, down-regulation of the nutrient-sensing 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, 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 (HSU *et al.* 2003; LIN *et al.* 1997). 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 evolutionally 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 are lethal in most strains (VAN HEUSDEN and STEENSMA 2006). 14-3-3 proteins function as molecular chaperones and protein tethers, which have more than 200 interacting partners (KAKIUCHI *et al.* 2007). Most binding partners of 14-3-3 proteins contain one of the consensus sequence motifs $RSXpS/TXP$ and $RXXXpS/TXP$ (*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). Genome-wide 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 (BRUCKMANN *et al.* 2007; ICHIMURA *et al.* 2004). 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 CR, TOR and PKA-mediated longevity pathways.

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 non-dividing state referred as chronological life span (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 only survived about 3 days. Therefore, factors that extended the survival of the *cdc25-10* mutant at 38° might also extend survival of wild type (WT) cells in stationary phase (CLS). We first introduced a 2 μ -based yeast over-expression 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, whose over-expression 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. WT (BY4742) cells over-expressing 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 over-expressions 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 WT 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). Deletion of *BMH1* also extended the maximum CLS in a different strain, W303 (Figure 1E), indicating 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 entered stationary phase. Shifting cells to water has been suggested to mimic the adverse growth conditions that yeast cells frequently encounter in wild 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 H₂O), 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 stress response (FABRIZIO and LONGO 2003; POWERS *et al.* 2006). As shown in Figure 2A (top panel), the *bmh1*Δ mutant exhibited increased resistance to heat stress to a level similar to that observed in CR cells, and the long-lived *tor1*Δ and *cdc25-10* mutants (Figure 2B). Interestingly, Bmh1-oe did not show increased resistance (Figure 2A, bottom panel), 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 stress response.

The heat shock factor HSF-1 has been shown to be required for starvation-induced life span extension as well as responding to heat shock and oxidative stress 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 heat shock elements (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 stress-sensing transcription factors Rim15 and Msn2/4 (VAN HEUSDEN and STEENSMA 2006) and to 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, Rim15 abolished *bmh1* Δ -induced resistance to heat stress. Furthermore, deleting Msn2/4 also abolished *bmh1* Δ -induced CLS extension (supplemental Figure 1), suggesting these stress response factors played important roles in *bmh1* Δ -induced heat resistance and CLS extension.

Intracellular homeostasis of reactive oxygen species (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 (H₂O₂) and paraquat (generating superoxide anions). As shown in Figure 2E, the

bmh1Δ mutant showed ~10-fold increased resistance to H₂O₂ compared to WT. In addition, *bmh1Δ* also conferred resistance to paraquat-induced toxicity (Figure 2F). These results demonstrate that deleting Bmh1 protects cells from oxidative stress. We next compared activities of superoxide dismutases (SOD) in cell extracts of the *bmh1Δ* mutant and WT cells using a SOD gel assay (RAYCHAUDHURI *et al.* 2003). Both *bmh1Δ* and WT showed higher Sod1 activities (cytosolic Cu/Zn-SOD) after entering stationary phase (Day 2 and 3)(Figure 2G). Interestingly, WT cells failed to show increased Sod2 activities (mitochondrial Mn-SOD) whereas the *bmh1Δ* mutant showed slightly higher Sod2 activities, suggesting Sod2 might play an important role in *bmh1Δ*-induced stress response. Finally, we determined the level of ROS in the *bmh1Δ* mutant using a ROS-specific fluorescence dye (ZUIN *et al.* 2008). Similar to CR treated cells, and the *tor1Δ* and *cdc25-10* mutants, the *bmh1Δ* mutant showed lower ROS level compared to WT (Figure 2H). These data suggest that *bmh1Δ* activates 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 *bmh1Δ* on CR and *tor1Δ* and *cdc25-10* induced CLS extension to determine whether *bmh1Δ* functioned in these pathways. Figure 3A showed that *bmh1Δ* further extended CR-induced CLS, suggesting *bmh1Δ* and CR functioned in parallel or partially overlapping pathways to extend CLS. Figure 3B and Figure 3C showed that *bmh1Δ* did not significantly further extend CLS of the *tor1Δ* and *cdc25-10* mutants suggesting *bmh1Δ* might function in the TOR and PKA pathways to extend CLS.

Since *bmh1Δ* further extended CR-induced CLS (Figure 3A), it was possible that *bmh1Δ* activated additional stress-response factor(s) to extend CLS of cells grown in CR. Deleting this *bmh1Δ*-specific stress response factor(s) should only affect *bmh1Δ*-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 *bmh1Δ* pathway. Our genetic studies suggested that *bmh1Δ* and *tor1Δ* might function in the same pathway to extend CLS (Figure 3C), we therefore examined whether *Gcn4*, a downstream target of TOR, might play a role in *bmh1Δ*-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 *gcn4Δ* specifically abolished *bmh1Δ*- and *tor1Δ*-induced but not CR-induced heat resistance. Furthermore, *Gcn4* was also required for *bmh1Δ*-induced CLS extension (Figure 3F). These results suggest that *Gcn4* is an important factor in the *bmh1Δ* pathway, which works in concert with other factors such as *Msn2/4* and *Rim15* to regulate 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 5A, B and C, the level of phosphorylated Bmh1 proteins increased ~3 to 4 fold in WT cells upon entering stationary phase (48 h). It was possible that phosphorylated Bmh1-Ser238 caused inhibitory effects on CLS since deleting Bmh1 extended CLS. In addition, both *tor1Δ* (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 *tor1Δ*, and that CR and *tor1Δ* 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 Bmh1, we screened 132 putative protein kinase mutants (defined in the *Saccharomyce cerevisiae* genome database, SGD) for kinases involved in phosphorylation of Bmh1-Ser238 (Supplemental Table 1) using the yeast non-essential 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 immuno-precipitated 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

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 over-expressions extended cell survival in an accelerated cell

death assay (Figure 1A) and chronological life span (CLS) (Figure 1B). Interestingly, we discovered that Bmh1 deletion also extended CLS (Figure 1C). Since Bmh1-oe only extended CLS 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 2E and 2F). Deleting Bmh1 also increased heat shock elements (HSE)-mediated transcription (Figure 2C). In addition, cells lacking Bmh1 had 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 protect cells from ROS-induced damages during chronological aging.

In line with our findings, RNAi-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 only extended CLS in cells grown and kept in SD but failed to extend CLS in cells shifted to H₂O (Figure 1B and 1G). It is possible that Bmh1-oe only extends CLS in cells that are more metabolically active. It has been shown that cells remain at 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 wild environment that yeast cells frequently encounter (FABRIZIO and LONGO 2003; GRAY *et al.* 2004). Over-expressions 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 14-3-3 proteins 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 *tor1Δ* (Figure 5B). Although *cdc25-10* did not decrease the level of phosphorylated 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 components of the autophagy pathway (KAKIUCHI *et al.* 2007). Our data showed that *bmh1Δ* required these components for heat resistance (Figure 2D) and/or CLS extension (supplemental Figure 1), suggesting the ability to respond to various types of metabolic and oxidative stresses was essential for *bmh1Δ*-mediated CLS extension. It is possible that during chronological aging, increased amount of phosphorylated Bmh1-Ser238 might enhance the ability of Bmh1 to sequester and/or interfere the interactions of these stress response factors with their interacting

partners. In line with 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 3B and 3C). We also showed that both TOR and PKA could affect Bmh1-238 phosphorylation (Figure 5A and 5E). 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 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, which 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 (DARLING *et al.* 2005; WILKER and YAFFE 2004). 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 the 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 play important roles in these diseases as well as life span regulation in mammals.

MATERIALS AND METHODS

Yeast strains and media: Yeast strains 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 α ura3-1, leu2-3, 112 his3-11, 15 trp1-1 ade2-1 RDN1::ADE2 can1-100* was 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 was minimal synthetic SD supplemented with 4x 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 were listed in Table 1. Strains over-expressing Bmh1 or Bmh2 were made by integrating the *pADH1-BMH1* or *pADH1-BMH2* plasmid into the genome.

Genetic screen conditions: About 20,000 colonies, which represented ~5 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 up-shifted to non-permissive 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 additional 2 days. Cell patches that grew on the assay plates at 38° were excluded since 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. 4 cell patches were identified under these conditions. Plasmid DNA conferring the strongest survival was recovered from the corresponding master plate and re-transformed into *cdc25-10* mutant to confirm the phenotype. Sequencing analysis of this DNA fragment indicated that it contained both the *NDJ1* and *BMH2* genes. Each gene was cloned into an integrative vector driven by the *ADH1* promoter, pPP81. Only *BMH2* over-expression extended survival.

Plasmid constructions: Bmh over-expression constructs p*ADH1-BMH1* and p*ADH1-BMH2* were made as follows: specific oligonucleotides were designed (with a *Not1* site added to the 5' end and a *Nhe1* site to the 3' end) to amplify the *BMH1* (or *BMH2*) coding region via PCR using *Pfu* polymerase. Amplified DNA was digested with *Not1* and *Nhe1* and then ligated to pPP81 (an integrative vector carrying a *LEU2* auxotrophic marker and an *ADH1* promoter).

Chronological Life Span (CLS): 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 OD₆₀₀ 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 h). 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 re-growth phenomenon was often observed: after ~90-99% of cells died, the number of viable cells increased. This re-growth phenomenon has also been reported in several other strains (FABRIZIO *et al.* 2004), and was likely due to nutrients released by dead cells and increased adaptive mutations during prolonged culture (FABRIZIO *et al.* 2004). We found that the re-growth problem could be alleviated by transferring 10-fold less cells to water at a final density of OD₆₀₀ ~1 (10⁷ cells/mL, ~10⁸ 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 (CR) for 2 days with starting OD₆₀₀ of 0.1 prior to analysis. For heat shock studies, cells were spotted onto YPD plates (2% glucose) in 5-fold serial dilutions (started at OD₆₀₀ 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 hydrogen peroxide toxicity test, cells were spotted onto YPD plates (with 0 or 3 mM H₂O₂) in 5-fold serial dilutions and were allowed to grow for 2 days. For paraquat toxicity test, cell growth was monitored in SD containing indicated concentrations of paraquat with starting OD₆₀₀ of 0.05 after incubation at 30° for 16 h.

ROS detection: Cells grown in minimal SD to stationary phase were washed twice with PBS buffer (PH 7.4) then resuspended in PBS with 10 μM H₂DCFDA following by 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 (~5x10⁶ cells/well). Fluorescence signals were detected using a plate reader with excitation at 485 nm and emission monitored at 535 nm.

SOD 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 SOD-active bands appeared white in a dark-blue background on gel. Results shown in Figure 2G were 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, 5µL 10X *Pfu* buffer, 1 µL p*ADH1*-Bmh1 plasmid DNA, 2 µL *Pfu* Turbo polymerase, and 1µL of each pair of oligos: S189A-f (5'-GAAATTCAAACCGCTCCAGAC-3') + S189A-r (5'-GTCTGGGAGCGTTTTGAATTTC-3') or S238A-f (5'-CAGACATGGCCGGAGTCCGGTC-3' + S238A-r (5'-

GACCGGACTCGGCCATGTCTG-3'). The PCR products were digested with *DpnI* then introduced into *XLI-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 keyhole limpet hemocyanin (KLH) conjugated phospho-peptides, SVFYIEIQN(p)SPDKAC (flanking Ser189) or TLWTSDM(p)SESGQAEDQ (flanking Ser238), by Antagene (CA). Antibodies were purified from the resulting antiserum by column chromatography on phosphopeptides conjugated affinity resin.

Protein extraction and Western blot analysis: Total protein extract was obtained as described (EASLON *et al.* 2008). About 15 μ g 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 antisera (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.

CIP Treatment: 250 mL cells ($\sim 5 \times 10^9$) grown in SD to mid-log phase were harvested and resuspended in 500 μ L 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. 7 μ L monoclonal anti-Myc antibody (Covance) was used to immuno-precipitate Myc epitope-tagged Bmh1 in 400 μ L cell

extract. For CIP (calf intestinal alkaline phosphatase) treatments (Ai *et al.* 2002), the immunocomplex was spun down, washed, then resuspended in 200 μ L CIP buffer (100 mM NaCl, 50 mM Tris-HCl, 10 mM MgCl₂, 1 mM DTT, pH 7.9). 100 μ L of this suspension was incubated with CIP (20 units, Roche) for 20 min at 37°. About 30 μ L 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 OD₆₀₀ ~1 in a total volume of 100 mL and induced for 2 hours 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: 15 mL cells were harvested at OD₆₀₀ ~10 and resuspended in 500 μ L breaking buffer: 50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 1 mM EDTA, 0.5% Triton X-100, 1 mM PMSF. Cell suspension was lysed by agitations using glass beads and the FastPrep beads beater. 5 μ L monoclonal anti-HA antibody (Covance) was used to immuno-precipitate HA epitope-tagged Bmh1 in 400 μ L cell extract. After CIP treatment, beads were washed with buffer (50 mM Tris-HCL pH 7.5, 100 mM NaCl, 0.2% Triton X-100) to remove CIP in the reaction, which were then resuspended in kinase buffer (20 mM Tris-HCl pH 7.5, 20 mM β -glycerophosphate, 100 μ M orthovanadate, 10 mM MgCl₂, 1 mM DTT, 50 μ M ATP, 1 mM NaF). 30 μ L recombinant Tpk1 (0.1 μ g/ μ L) was added to initiate the kinase reaction at 30° for 30

min.

Heat shock Element (HSE) 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 w/o URA at 30° with starting OD₆₀₀ 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.

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TABLE 1: Yeast strains used in this study.

Strain	Genotype	Reference
W303AR	<i>MATa ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1</i> <i>RDN::ADE2 can1-100</i>	(LIN <i>et al.</i> 2000)
LSC1041	W303AR <i>bmh1Δ::Kan^r</i>	This study
LSC14	W303AR <i>cdc25-10</i>	(LIN <i>et al.</i> 2000)
BY4742	<i>MATa his3Δ1 leu2Δ0 lysΔ0 ura3Δ0</i>	(EASLON <i>et al.</i> 2007)
LSC722	BY4742 <i>bmh1Δ::Kan^r</i>	This study
LSC716	BY4742 <i>bmh2Δ::Kan^r</i>	This study
LSC622	BY4742 p <i>ADH1-BMH1-LEU2</i>	This study
LSC623	BY4742 p <i>ADH1-BMH2-LEU2</i>	This study
LSC89	BY4742 p <i>ADH1-XXX-LEU2</i> (pPP81, vector control)	(EASLON <i>et al.</i> 2007)
LSC586	BY4742 <i>tor1Δ::Kan^r</i>	This study
LSC157	BY4742 <i>cdc25-10</i>	(EASLON <i>et al.</i> 2007)
LSC837	BY4742 <i>cdc25-10 bmh1Δ::Kan^r</i>	This study
LSC801	BY4742 <i>tor1Δ bmh1Δ::Kan^r</i>	This study
LSC1021	BY4742 <i>bmh1Δ::Kan^r</i> p <i>ADH1-Bmh1-S189A-LEU2</i>	This study
LSC1020	BY4742 <i>bmh1Δ::Kan^r</i> p <i>ADH1-Bmh1-S238A-LEU2</i>	This study
LSC327	BY4742 <i>msn2Δmsn4Δ::Kan^r</i>	This study
LSC971	BY4742 <i>msn2Δmsn4Δbmh1Δ::Kan^r</i>	This study
LSC516	BY4742 <i>rtg3Δ::Kan^r</i>	This study
LSC997	BY4742 <i>bmh1Δrtg3Δ::Kan^r</i>	This study
LSC1246	BY4742 <i>gnc4Δ::Kan^r</i>	This study
LSC1245	BY4742 <i>bmh1Δgcn4Δ::Kan^r</i>	This study
LSC1244	BY4742 <i>tor1Δgcn4Δ::Kan^r</i>	This study
LSC1218	BY4742 <i>rim15Δ::Kan^r</i>	This study
LSC1219	BY4742 <i>bmh1Δrim15Δ::Kan^r</i>	This study

FIGURE LEGENDS

FIGURE 1. -Characterization of yeast 14-3-3 proteins as novel longevity factors that regulate chronological life span (CLS). (A) Over-expression of clone #1 (containing *BMH2*) extends survival in an accelerated cell death assay. Temperature sensitive *cdc25-10* mutants carrying a control 2 μ vector or clone #1 are first incubated at 38° for 4 days and then are incubated at 30° for 2 days. About 5x10⁴ cells are spotted in the first column (5x). (B) Bmh1 and Bmh2 over-expressions extend CLS of cells grown and kept in SD. (C) *bmh1* Δ extends 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 WT and *bmh1* Δ and *bmh2* Δ mutant cells are determined by measuring mutation frequency of the *CAN1* gene. Stationary phase cells (day 4 in SD) are collected then plated onto both YPD and SD-ARG (w/o arginine, containing 60 mg/L L-canavanine sulfate). Mutation frequency is calculated by normalizing the number of colonies appeared on SD-ARG to that of corresponding YPD. For each strain, 12 independent colonies are examined. (E) *bmh1* Δ extends maximum CLS in a different strain W303. (F) *bmh1* Δ extends CLS of cells shifted to water. Cells are first grown in SD to stationary phase then are shifted to sterile water. (G) Bmh1 and Bmh2 over-expressions do not extend CLS of cells shifted to water. (H) *bmh1* Δ extends both mean and maximum CLS. Results show statistics of 11 independent experiments each conducted in triplicate or quadruplicates. Each symbol represents average viability of 4 samples each containing 10⁷ cells. For CLS analysis, one representative set of three independent experiments, each conducted in quadruplicate, is shown. “Days” denotes the number

of day cells in SD or H₂O. Error bars denote standard deviations. *P* values are calculated using the Student's *t*-test (*: *P*<0.05; **: *P*<0.01). WT: wild type control; v: vector control; oe: over-expression.

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 panel). Bmh1-oe does not increase heat resistance (bottom panel). Results show 5-fold 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 heat shock elements (HSE)-driven transcription activity. Results show WT and *bmh1*Δ mutant cells (carrying a HSE-driven β-galactosidase reporter plasmid) grown on YPD containing 4 mg X-gal. Dark blue color shown in the *bmh1*Δ mutant grown at 37°C indicates higher HSE-driven transcription activity. (D) *bmh1*Δ-induced heat resistance requires Msn2/4 and Rim15. (E) *bmh1*Δ confers resistance to H₂O₂-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 pre-grown in SD with 0.5% glucose prior to analysis.

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) *bmh1Δ* does not significantly affect *tor1Δ*-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 day cells in H₂O. One representative set of three independent experiments, each conducted in quadruplicate, is shown. (D) CR-induced heat resistance requires stress response factors Msn2/4, Rtg3 and Rim15. (E) Deleting *GCN4* totally abolishes *bmh1Δ*-induced heat resistance. (F) *bmh1Δ*-induced increase in survival requires Gcn4. Results show cell viability determined in day 6 cultures (4 days after shifting to H₂O). (G) A proposed model for *bmh1Δ*- and CR-induced CLS extension. *bmh1Δ* 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 *bmh1Δ* 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 pre-grown in SD with 0.5% glucose prior to analysis.

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 *bmh1Δ* and *bmh2Δ* mutants are analyzed. About 10 μL cell extract is loaded in each lane (for Figure A, B, C and E). (B) The anti-Bmh1-total antibody specifically recognizes Bmh1 proteins. (C) Levels of protein expression in cells over-expressing 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 immuno-precipitated Myc-tagged Bmh1. CIP: calf intestinal alkaline phosphatase; IP: immuno-precipitation. (E) The anti-Bmh1-pS238 antibody is specific to phosphorylated Ser238 of Bmh1. Total protein extracts of WT and *bmh1*Δ cells expressing mutated Bmh1-S238A or Bmh1-S189A are analyzed. WT: wild type control; oe: over-expression.

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 at the bottom panels indicate relative amount of Bmh1-pS238 normalized to the levels of Bmh1-pS238 at 8h. (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. For (A) to (D), Results show duplicated blots each loaded with same amount of proteins from the same sample in each lane. For (A) to (C), the amount of Bmh1-pS238 is first normalized to internal loading control then normalized to the levels of Bmh1-pS238 at 8h. (E) Recombinant Tpk1 can phosphorylate Bmh1 at Ser238 *in vitro*. WT: wild type control; CR: 0.5% glucose. (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 remains possible that

phosphorylation of Bmh1 at other residues also play important roles in life span regulation, and that non-phosphorylated Bmh1 may also induce certain beneficial effects on life span.









