Studies on the regulatory mechanism of cell growth and life span in budding yeast

出芽酵母の細胞増殖と寿命の制御機構に関する研究

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(2) Implication of Ca\(^{2+}\) in the regulation of replicative life span of budding yeast.
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INTRODUCTION

To adapt to various stresses and changes of external environment, cells operate the response mechanisms appropriate to these environmental changes. This adaptation performed through many signal pathways controls cell morphology and cell growth by the activation of transcription of required genes or enzymes. The mechanisms of each signaling pathways are highly conserved to many eukaryote: the enzyme group activated by second messenger such as cAMP or Ca^{2+}, and MPAK (Mitogen-activated protein kinase) pathways.

cAMP is discovered as an intracellular mediator of a hormonal function, and later analyses have showed that this molecule acts as a second messenger used for intracellular signal transduction in many different organisms (for review, see Joseph et al., 2002). PKA (Ras/cAMP-dependent Protein Kinase), which is the phosphorylating enzyme activated by cAMP, phosphorylates substrate proteins and changes its activity. PKA pathway has been identified as a regulator of a wide variety of physiology mechanisms such as cell cycle progression, stress response, differentiation, and life span (for reviews, see Matsumoto et al., 1985; Thevelein et al., 1999; Santangelo et al., 2006). In the mammals, this pathway has an important role in the signal transduction through many hormones, such as adrenalin and glucagon. In the budding yeast *Saccharomyces cerevisiae*, most signal transductions induced by glucose, major carbon source in a medium, are mediated by PKA pathway. Sufficient external glucose activates adenylate cyclase, which catalyzes the conversion of ATP to cAMP, and increases the level of cAMP. Activation of PKA by an increased cAMP results in the progression of cell cycle and the activation of growth (for reviews, see Matsumoto et al., 1985; Thevelein et al., 1999; Santangelo et al., 2006). Although the varied physiological roles of cAMP/PKA pathway have been clarified, a few substrates/targets of PKA have been identified.

In eukaryotic cells, intracellular Ca^{2+} level, which is usually kept low, is transiently increased by various stimuli, and this increase in cellular Ca^{2+} induces activation of a T-cell, discharge of muscle contraction and neurotransmitter (for reviews, see Carafoli et al., 2002; Berridge et al., 2003). The Ca^{2+}/calmodulin-dependent protein phosphatase calcineurin (CN) is conserved from yeast to human, and is involved in various cellular processes including immune response and extension of a nerve axon in higher organisms. Budding yeast CN has been implicated in stress-induced gene expression,
ion homeostasis, cell cycle regulation, and maintenance of viability after exposure to the mating pheromone (Aramburu et al., 2000; Cyert et al., 2001; Miyakawa et al., 2007). We showed previously that both Ca\textsuperscript{2+}-signaling pathways, Mpk1 MAP kinase and CN, control G\textsubscript{2}/M transition by regulating Swe1, a negative regulator of the Cdc28-Clb complex (Mizunuma et al., 1998). A recent research reported that the metabolic disorder of Ca\textsuperscript{2+} has occurred in a mouse with the phenotype similar to a human aging (Imura et al., 2007), and it was shown that CN and AMPK regulated life span cooperatively in a nematode (Dwivedi et al., 2009; Mair et al., 2011). Although it is suggested that Ca\textsuperscript{2+}-signaling pathways is involved in the regulation of life span, the relationship between Ca\textsuperscript{2+} and aging in yeast is not known.

Because both cAMP and Ca\textsuperscript{2+} are highly conserved from yeast to higher eukaryotes, it is very useful to clarify the signaling pathways activated by these molecules in budding yeast as a model organism. Moreover, these highly conserved relationships between yeast and mammalian cells can be expanded to the drug developments. Indeed, the immunosuppressive drugs such as FK506 and rapamycin inhibits the same targets molecule in human and yeast, and it has been reported that the mechanism of action is also similar.

The purpose of this study was to investigate the regulatory mechanism of cell growth mediated by Ca\textsuperscript{2+} signaling pathway in budding yeast. The results obtained are presented in two main parts. In the first part, I focus on Whi3, an RNA binding protein. The mutant allele of \textit{WHI3} was identified as a suppressor mutation of the Ca\textsuperscript{2+}-sensitive phenotypes of \textit{Δhog1} cells. Hog1 is a mitogen-activated protein kinase (MAPK) integral to the osmo-regulatory signal transduction cascade (HOG signaling pathway), which affects various gene expressions in response to hyperosmotic stress. I describe the identification of PKA as an upstream regulator of Whi3 involved in a variety of physiological functions, and show that the phosphorylation of Ser-568 in Whi3 by PKA played an inhibitory role in Whi3 function (CHAPTER I). In the second part, I investigate the relationship between Ca\textsuperscript{2+}-signaling and life span. The strain lacking Zds1, a negative regulator of Swe1, shows G\textsubscript{2} cell cycle arrest, and polarized bud growth, due to the activation of the cellular Ca\textsuperscript{2+}-signaling pathways. Here I show that this Ca\textsuperscript{2+}-signaling activated cells had decreased life span as well as the shortened life span by increased external Ca\textsuperscript{2+}. On the other hand, the deletion of \textit{CNB1} encoding the regulatory subunit of calcineurin promoted aging, indicating that appropriate calcineurin
activity plays an important role in a longevity (CHAPTER II). This study clarified the relationship between regulators important for cell growth control and the regulation of life span.
CHAPTER I

Ras/cAMP-dependent Protein Kinase (PKA) Regulates Multiple Aspects of cellular Events by Phosphorylating the Whi3 Cell Cycle Regulator in Budding Yeast
1.1. ABSTRACT
Eukaryotic cells monitor internal and external signals to commit themselves to a new mitotic cycle or to diverse differentiation processes in the G1 phase of the cell cycle. In the budding yeast *Saccharomyces cerevisiae*, this control network is called Start. The Start/G1 phase is initiated by the G1 cyclin Cln3, which associate with the cyclin-dependent kinase Cdc28. Previously, we identified a mutant allele of *whi3*, a negative regulator of the G1 cyclins, as a suppressor mutation of the Ca^{2+} or osmotic stress sensitive phenotype of *hog1*-delted cells (*sgh* mutants). Whi3 was originally identified as a positive regulator of cell-size control and is involved in the regulation of Start. The best characterized function of Whi3 is its ability to inhibit the Cln3 function in the G1 phase by binding to *CLN3* mRNA, thereby inhibiting the expression of the G1 cyclin *CLN1* and *CLN2*. In addition, Whi3 functions as the regulator of developmental options such as invasive growth and meiosis. However, the regulatory pathway of Whi3 in response to multiple signals remains largely unknown. In this study, I describe the identification of the RAS/cAMP-dependent protein kinase (PKA) as the Whi3 kinase. Whi3 was phosphorylated by PKA and that the phosphorylation of Ser-568 in Whi3 by PKA played an inhibitory role in the Whi3 function. This mechanism was required for the promotion of the G1/S progression. Further, I demonstrate that the phospho-mimetic S568D mutation of Whi3 prevented the developmental fate switch to sporulation or invasive growth. Moreover, both phospho-mimic (Whi3-S568D) and hyper-phosphorylated forms of Whi3 (in the *bcy1* cells) reduced interaction with *CLN3* mRNA. Thus, PKA modulated the function of Whi3 by phosphorylation, and implicating PKA-mediated modulation of Whi3 in multiple cellular events.

1.2. INTRODUCTION
Eukaryotic cells evaluate and cope with their external environment by using rapidly responding, various complex signaling pathways. Extracellular stresses activate the stress-activated protein kinases (SAPKs) that regulate transcription levels of genes required for adaptation to stresses (Wilkinson et al., 1998). The activated SAPKs result in transient cell cycle arrest until the cells have adapted to their new environment. This adaptation system is important for resumption of the cell cycle.
In the budding yeast *Saccharomyces cerevisiae*, the Hog1 SAPK pathway mediates adaptation to the condition of high osmolarity (Brewster *et al*., 1993). The mechanism activating the HOG pathway is well understood (for review, see Hohmann *et al*., 2002). Hog1 is also required for cell cycle regulation after exposure to hyperosmotic stress (Alexander *et al*., 2001; Belli *et al*., 2001; Clotet *et al*., 2006). For instance, activating Hog1 induces a G1 cell cycle arrest by a dual mechanism that involves down-regulation of the G1 cyclin transcription and direct phosphorylation of the CDK-inhibitor Sic1 (Escoté *et al*., 2004). Moreover, we previously showed the evidence for antagonistic regulation of cell growth by the calcineurin-Crz1 pathways activated by Ca2+ and the Hog1 pathways (Shitamukai *et al*., 2004). However, the relation between the Ca2+ signaling pathways and the Hog1 pathways still largely remain to be investigated.

The growth of Δhog1 cells is compromised on solid medium containing a high concentration of Ca2+, exhibiting hyperpolarized bud growth and G2 cell-cycle delay (Shitamukai *et al*., 2004; Kobayashi *et al*., 2008). To identify the genes involved in this regulatory mechanism, we screened for mutants that suppressed the growth defect of hog1-deleted cells under high Ca2+ condition. The isolated mutants were classified into 6 loci designated as sgh1-sgh6 (for suppressors of Ca2+-induced growth defect of hog1). Of these mutants, we identified the mutant allele of WHI3 as sgh4.

Whi3 is characterized as a RNA binding protein. It was originally identified as a positive regulator of the cell size control required for passage through Start. The main function of Whi3 is suppression of the G1 cyclin *CLN1, CLN2* level by binding to *CLN3* mRNA. Thereby, Whi3 inhibits the association between G1 cyclin and Cdc28, leading to the activation of two transcription factors SBF (Swi4-Swi6) and MBF (Mbp1 and Swi6), which activate the expression of the G1 cyclin *CLN1, CLN2* mRNAs. The G1/Start phase in the cell cycle is important for the determination of their developmental fates: beginning of mitotic progression, or the switch to alternative stages (Hartwell *et al*., 1974). Whi3 ensures reversible cell-cycle entry through the inhibition of the Start progression if conditions later become unfavorable (Gari *et al*., 2001; Sudbery *et al*., 2002; Wang *et al*., 2004; Colomina *et al*., 2008). Indeed, it is suggested that Whi3 function is required for developmental options such as invasive growth and meiosis (Gari *et al*., 2001). However, little is known about the evidence for an upstream regulator(s) of Whi3.
Protein phosphorylation, one of the post-translational modifications, plays a pivotal role in the regulation of various proteins by altering their function and activity. The RAS/cAMP-dependent protein kinase (PKA) is the phosphorylating enzyme activated by cAMP. PKA pathway in yeast has several functions, including glucose metabolism, stress response, cell growth, aging, and autophagy (for review, see Matsumoto et al., 1995; Thevelein et al., 1999; Santangelo et al., 2006). The molecular mechanism by which yeast cells sense and respond to the specific stimuli activated by PKA has been elucidated. In addition, it has been shown that PKA activity is important for the transcriptional regulation of the G₁ cyclins (CLN₁, CLN₂), and the regulation of the critical cell size required for Start in response to nutrient conditions (for review, see Matsumoto et al., 1995; Thevelein et al., 1999; Santangelo et al., 2006). However, despite the obvious importance of this signaling pathway, only a few PKA substrates important for the cell growth have been identified.

In this chapter, I show that PKA regulates Whi3 functions. I describe that the phosphorylation of Serine-568 in Whi3 by PKA played an inhibitory role in Whi3 functions. This Whi3 phosphorylation accelerated the G₁/S progression. Further, I demonstrate that the phospho-mimic S568D mutant of Whi3 failed sporulation and invasive growth. Based on these results, I propose that the phosphorylation of Whi3 by PKA was involved in multiple cellular events, including cell-cycle control and developmental fate in response to environmental stimuli.

1.3. MATERIALS AND METHODS
1.3.1. Yeast Strains and Media
Yeast strains used in this study are listed in Table 1. All yeast strains were derivatives of W303. The media used were as described previously (Mizunuma et al., 1998).

1.3.2. Site-Directed Mutagenesis and Construction of Plasmids
The pMBP-WHI3 plasmid containing the fusion gene for the maltose-binding protein (MBP)-Whi3 conjugate protein was constructed as follows: The WHI3 gene was amplified by PCR, digested with BamHI and Sall, and then cloned into the BamHI- and Sall- digested pMAL-C2 vector (provided by A. Kikuchi). The pMBP-WHI3-S568A plasmid containing the fusion gene for the mutant MBP-Whi3 S568A conjugate protein
was constructed by using a QuikChange™ XL Site-Directed Mutagenesis Kit (STRATAGENE) and the pMBP-WHI3 plasmid as a PCR template. The pWhi3-S568A-3HA plasmid was constructed as follows: First, the BamHI-SalI fragment of the pMBP-WHI3-S568A plasmid was cloned into BamHI- and -SalI-digested pUC119 to construct pWhi3-S568A. Next, the WHI3 gene containing a 3HA epitope tag was amplified from genomic DNA of the WHI3-3HA::kanMX6 strain (provided by Dr. M. Aldea) by PCR and cloned into the T7Blue T-Vector (Novagen) to construct pT-Whi3-5T-3HA. Finally, the ApaI-SphiI fragment of the pT-Whi3-5T-3HA plasmid was cloned into the ApaI- and SphiI- digested pWhi3 S568A plasmid. The pWhi3-S568D-3HA plasmid was constructed similarly by using the pWhi3-S568A-3HA plasmid as the PCR template. The mutations were confirmed by DNA sequencing.

1.3.3. Gene Disruption and Strain Construction

The ∆whi3 strain on the W303 background was constructed by gene replacement. Genomic DNA was isolated from the ∆whi3::kanMX4 strain on a BY4741 background (Invitrogen). The PCR-amplified fragments of ∆whi3::kanMX4 were used to transform the W303-1A and MLY41a strains (Longtine et al., 1998). Deletion of the genomic CLN3 gene (CLN3 plasmids provided by I. Yamashita) and the BCY1 gene was performed by using a disruption plasmid.

The strains with a chromosomally integrated gene for WHI3-S568A and WHI3-S568D with a 3x HA epitope at their C terminus were constructed as follows: The pWhi3-S568A-3HA and pWhi3-S568D-3HA plasmids were digested with NspV (within the WHI3 locus) and used to transform the appropriate strains. Insertion of these fragments into the original WHI3 locus was confirmed by DNA sequencing and Western blot analysis.

1.3.4. In Vitro Phosphatase Assay

The cell extracts prepared as described above (200 μg total protein) were incubated with 400 U of λ-phosphatase (New England Biolabs) in 50 μl of λ-phosphatase buffer and 1 mM MnCl₂ with or without phosphatase inhibitors (50 mM NaF and 5 mM Na₃VO₄) for 60 min at 30°C. After the phosphatase treatment, 4 x sample buffer for SDS-PAGE was added; and the mixture was then boiled for 10 min. Whi3-HA was
detected by immunoblotting.

1.3.5. **In Vitro Protein Kinase Assay**

The procedure for the *in vitro* kinase assay was carried out as described previously (Zhu *et al.*, 2000). Expression of the MBP-Whi3 fusion protein in *E.coli* BL21 was induced by the addition of 0.1 mM IPTG to the culture medium. MBP-Whi3 protein was affinity-purified by use of amylose resin beads (New England Biolabs) according to the manufacturer's instructions. Protein kinases from a collection of 119 yeast protein kinases fused to glutathione S-transferase (kindly provided by M, Snyder) (Zhu *et al.*, 2000) were over-expressed in yeast and affinity-purified by using glutathione-Sepharose 4B beads (Amersham Biosciences). The MBP-Whi3 and GST-fused protein kinases on the beads were mixed in 50 μl of kinase buffer containing 0.5 μCi of [γ-32P] ATP, 100 μM ATP, and 10 mM MgCl₂ and incubated at 30°C for 30 min. After the beads had been washed with the kinase buffer, the proteins were eluted by boiling the beads in SDS-sample buffer for 5 min. The eluted proteins were resolved by SDS-PAGE and detected by autoradiography.

1.3.6. **Invasive Growth Assay**

Yeast cells (MLY41a; provided by J. Heitman) were streaked onto standard medium (YPD; 1% yeast extract, 2% peptone, 2% glucose) plate and allowed to grow at 28°C for 2 days. The plate was observed before and after rinsing with a gentle stream of water to remove all the cells from the agar surface.

1.3.7. **Sporulation Assay**

To induce sporulation, cells grown in YPD were shifted to YPAc (1% yeast extract, 2% peptone, 2% potassium acetate), grown for at least 3 generations at 28°C, and harvested at a density of 3-5 x 10⁷ cells/ml. The cells were washed twice with sporulation medium (SPM; 1% potassium acetate), resuspended in the same medium to 1.5 x 10⁷ cells/ml, and further incubated at 28°C. The formation of asci was assessed by phase-contrast microscopy.

1.3.8. **RNA Binding, Immunoprecipitation, and mRNA Detection**

Exponentially growing cells (4 x 10⁸) were disrupted with glass beads in 200 μl of
extraction buffer (50 mM Tris-HCl (pH 7.5), 150 mM KCl, and 2 mM MgCl₂) containing 40 units/μl RNasin (Qiagen), phosphatase inhibitors (10 mM NaF and 1 mM Na₃VO₄), 0.1% Nonidet P-40, 1 mM DTT, 1 mM PMSF, 10 μg/ml aprotinin, and protease inhibitor mixture (α-Complete, Roche Applied Science). Extracts were cleared by centrifugation for 15 min at 14,000 x g. Anti-HA monoclonal antibody (HA.11, Berkeley Antibody Co.) and protein A-agarose beads were added to the cleared extracts, followed by incubation for 8 h at 4°C. The beads were then washed four times with wash buffer (50 mM Tris-HCl (pH 7.5) containing 150 mM KCl and 2 mM MgCl₂) and subsequently eluted for 10 min at 65°C in 50 mM Tris-HCl (pH 8.0) containing 100 mM NaCl, 10 mM EDTA, and 1% SDS. Eluted samples were extracted by use of an RNeasy MiniKit (Qiagen) following the manufacture’s instructions. RT-qPCR was performed with 2 μl of RNA as template, and was done in a LightCycler (Roche Applied Science) with a One Step SYBR PrimeScript RT-PCR Kit II (TaKaRa).

1.4. RESULTS

1.4.1. sgh4/whi3 Suppresses Osmosensitivity of Δhog1 Cells

The strain lacking HOG1 shows severe growth defect, which is accompanied by a G₂ delay and polarized bud formation, on medium containing high Ca²⁺ due to the activation of the cellular Ca²⁺-signaling pathways and the high osmolarity (Shitamukai et al., 2004; Kobayashi et al., 2008). To clarify this regulatory mechanism of the cell cycle and the growth control regulated by Ca²⁺, we screened for suppressor mutants of the Ca²⁺-sensitive phenotypes of hog1 deletion cells. The isolated mutants were classified into 6 loci designated sgh1-sgh6 (for suppressors of Ca²⁺-induced growth defect of hog1). By gene cloning and genetic linkage analysis, the sgh1, -2, -3, -5, and -6 mutations have been identified as the mutant alleles of the SWE1, CNB1, TUP1, SKO1 and CYC8 gene, respectively (Kobayashi et al. 2008). Although these mutations were originally isolated by the ability to suppress the CaCl₂ sensitivity of the Δhog1, the 4 mutations (sgh3/tup1, sgh5/sko1, sgh6/cyc8, and sgh4), but not 2 mutations (swe1 and cnb1), suppressed also the osmo-sensitivity (0.6 M sorbitol and 0.4 M KCl) of the hog1 mutant (Kobayashi et al. 2008). Further, we have shown that the suppression by 3 mutations (sgh3/tup1, sgh5/sko1, and sgh6/cyc8) was caused by the defect in the Tup1-Cyc8 global repressor, which led to an altered expression of the genes involved in
osmotic stress responses (Kobayashi et al. 2008). In this chapter, I focused on the remaining mutation, sgh4.

By gene cloning and genetic linkage analysis, sgh4 was identified as mutant allele of the WHI3 gene, a negative regulator of the G_1 cyclin for cell size control (Nash et al. 2001). Disruption of the WHI3 gene, similarly as the sgh4 mutation, suppressed the Ca^{2+}-, and osmo-sensitive phenotype of Δhog1 cells, suggesting that the sgh4 mutation is a loss-of-function allele of the WHI3 gene (Fig. 1). Thus, I used the whi3-deletion mutant in further experiments.

1.4.2. Identification of PKA as the Whi3 Kinase

Whi3 was originally identified as a negative regulator of the G_1 cyclin for cell-size control (Nash et al. 2001) and shown to play an important role in regulating Cln3 activity in G_1 (Garí et al. 2001). Although the various physiological roles of Whi3 have been clarified, little is known about the upstream regulators of Whi3. Although the various physiological roles of Whi3 in multiple cellular events, including cell-cycle progression and developmental fate, the upstream regulator(s) of Whi3 has still not been fully identified. Protein phosphorylation, one of the post-translational modifications, plays a central role in the regulation of various proteins by altering their function and activity. As the phosphorylation of Whi3 has not been analyzed yet, I examined whether Whi3 could be phosphorylated in vivo. I detected slow-migrating bands of Whi3-HA on an SDS-PAGE gel (Fig. 2A, lane 1). The phosphatase-treated Whi3-HA protein migrated faster than the untreated protein (Fig. 2A, compare lanes 1 and 2), indicating that Whi3 was phosphorylated under the normal growth conditions.

To identify the protein kinase(s) responsible for the phosphorylation of Whi3, I screened for the kinase that was capable of phosphorylating the recombinant MBP-Whi3 fusion protein in vitro, using γ-^{32}P-ATP as the phosphate donor, from a collection of 119 potential protein kinases consisting of those previously characterized and predicted in the yeast genome database. Analysis of these kinases indicated that Tpk1, one of the 3 isoforms of the cAMP-dependent protein kinase (PKA), had the highest kinase activity for MBP-Whi3 but no activity was detected in MBP (Fig. 2B, lanes 1 and 2). The 2 other isoforms of PKA, i.e. Tpk2 and Tpk3, exhibited moderate kinase activity in this assay (Fig. 2B, lanes 3 and 4).

To identify the site(s) of Whi3 phosphorylated by PKA, I searched for the consensus
sequence for PKA-mediated phosphorylation (R(R/K)X(S/T)) (Ptacek et al. 2005) in Whi3 and found only one consensus sequence (Ser-568) located within the RNA-recognition motif (RRM) of Whi3 (Fig. 2C). To investigate whether Ser-568 is phosphorylated in vitro, I constructed a RRM of Whi3 that contained amino acids 539–621 and then constructed non-phosphorylatable mutant RRM-S568A recombinant protein, whose 568th Serine residue is substituted for Alanine, after which I compared the phosphorylation levels of these recombinant proteins. The phosphorylation level of MBP-RRM-S568A was statistically significantly reduced relative to that of MBP-RRM but to a modest extent (Fig. 2D), suggesting that Ser-568 of Whi3 is one of the phosphorylation sites mediated by PKA in vitro.

To confirm that Whi3 was phosphorylated by PKA in vivo, I examined whether the phosphorylation level of Whi3 would be elevated by the deletion of Bcy1, the regulatory subunit of PKA, in which PKA becomes constitutively active. As expected, in the Δbcy1 cells, the significant slow-migrating bands were observed, indicating the hyper-phosphorylation forms of Whi3-HA (Fig. 2E, lanes 1 and 2). The phosphatase-treated Whi3-HA protein in Δbcy1 cells migrated faster than the untreated protein (Fig. 2F, compare lanes 3 and 5), confirming that Whi3 had been phosphorylated by PKA in vivo. To examine whether Ser-568 is a phosphorylation site of Whi3 in vivo, I constructed the non-phosphorylatable mutation Whi3-S568A by site-directed mutagenesis and compared the phosphorylation levels of Whi3-HA and the Whi3-S568A-HA mutant protein in both WT and Δbcy1 cells (Fig. 2E). In the WT cells, the difference between the phosphorylation level of the Whi3-S568A-HA mutant protein and that of the Whi3-HA protein was not observed (Fig. 2E, lanes 1 and 3), suggesting that an additional phosphorylation site(s) of Whi3 mediated by PKA or another kinase(s) might exist in Whi3. In contrast, in the Δbcy1 cells, the major mobility shift in the Whi3-HA protein was not observed in the Whi3-S568A-HA mutant protein (Fig. 2E, lanes 2 and 4). These results indicate that Ser-568 of Whi3 is the site phosphorylated by PKA in vivo.

1.4.3. Phospho-mimic Whi3-S568D Mutation Decreases the Cell Size

The above results indicated that Ser-568 of Whi3 was one of the major phosphorylation sites recognized by PKA in vivo. So I focused on the role of this phosphorylation of Whi3 in multiple cellular events mediated by Whi3. As Whi3 was
originally identified as a positive regulator of cell-size control (Nash et al. 2001), I first examined whether the phosphorylation state of Ser-568 in Whi3 participated in the cell-size control. Wild-type (Whi3-HA) and the Whi3-S568A cells growing in normal YPD medium were similar in size. In contrast, the size of Whi3-S568D mutant cells, like that of the deletion mutant (Δwhi3), was smaller than that of the WT cells (Fig. 3). These results suggest that the phosphorylation of Whi3 by PKA resulted in the decreased cell size by inhibiting the Whi3 function.

1.4.4. Phosphorylation State of Ser-568 in Whi3 Affects the G1/S Transition by Modulating CLN2 Transcription

As the whi3 deletion promotes the G1/S transition by accelerating CLN2 transcription (Garí et al. 2001), I next investigated whether the PKA-mediated Whi3 phosphorylation participated in the cell cycle regulation. I examined the cell-cycle progression and CLN2 transcription of Whi3 mutant cells synchronized in the G1 phase. The Whi3-S568D mutation, like the deletion one (Δwhi3), led to a shortened G1 phase compared to the length of the G1 phase in WT cells (Fig. 4A). In contrast, the Whi3-S568A mutation led to a prolonged G1 phase (Fig. 4A). Consistent with the effect of these mutations on the cell-cycle progression, transcription of the CLN2 mRNA was accelerated by the Whi3-S568D mutation and decelerated by the Whi3-S568A mutation (Fig. 4B and C). These results suggest that the PKA-mediated Whi3 phosphorylation at Ser-568 inhibits the Whi3 function, thus promoting the start of the cell cycle.

1.4.5. Phosphorylation of Ser-568 in Whi3 Is Important for Cell Fate Determination

Whi3 is important for the inhibition of the Cln3 function in the G1 phase, leading to sporulation or invasive growth (Garí et al. 2001). Coincidentally, PKA has been shown to be required for the growth regulation to the switch to sporulation and filamentous growth (Thevelein et al. 1999). Thus, we examined the effects of the Whi3 mutations on sporulation and invasive growth. The haploid Whi3-S568D mutant, but not the Whi3-S568A mutant, was defective in invasive growth (Fig. 5A). Further, the homologous diploid Whi3-S568D mutant, but not the Whi3-S568A mutant, failed to sporulate (Fig. 5B). As it has been reported that defects in both sporulation and invasive growth of Δwhi3 cells are alleviated by the deletion of CLN3 (Garí et al. 2001), I
examined whether these deficiencies of the Whi3-S568D mutant were caused by promotion of the G1/S transition through the up-regulation of Cln3. As expected, both defects of the Whi3-S568D mutant were reversed by the CLN3 deletion (Fig. 5A and B). These results indicate that the PKA-mediated phosphorylation of Ser-568 in Whi3 plays key role in cell-fate determination in the G1 phase.

1.4.6. Phosphorylation of Whi3 by PKA Leads to Its Decreased Interaction with CLN3 mRNA

All of the above results are consistent with the idea that PKA-mediated phosphorylation of Whi3 at Ser-568 has inhibitory role in Whi3 function. How did PKA down-regulate it? Whi3 negatively regulates CLN1, CLN2, and CLN3 mRNA levels by binding to these mRNAs, most efficiently to CLN3 mRNA (Garí et al. 2001). Thus, I speculated that the phosphomimetic Whi3-S568D mutant and the hyperphosphorylated form of Whi3 in the bcy1 strain would show decreased interaction with mRNA. To examine this possibility, I carried out Whi3 immunoprecipitation followed by RT-qPCR analysis to measure Whi3 association with CLN3 mRNA. As expected, both the phosphomimetic (Whi3-S568D-HA) and hyperphosphorylated (Δbcy1 Whi3-HA) forms of Whi3 displayed reduced interaction with CLN3 mRNA in this assay (Fig. 6). Inversely, the Whi3-S568A mutant showed increased interaction with CLN3 mRNA (Fig. 6). Moreover, the increase in the ability of Whi3-S568A to bind to CLN3 mRNA was still observed in the Δbcy1 mutation background (see Δbcy1 Whi3-S568A mutant), although the binding ability was slightly reduced by the Δbcy1 mutation. This result indicates that the Whi3-S568A mutation is epistatic to the Δbcy1 mutation and further supports the possibility that Ser-568 of Whi3 is a major regulatory site phosphorylated by PKA for down-regulating the ability of Whi3 to bind to CLN3 mRNA. Altogether, these results suggest that PKA promotes G1/S progression by phosphorylating Whi3, which in turn causes reduced interaction with CLN3 mRNA.

1.5. Discussion

Whi3 function is important for not only control of the G1/S progression but also the switch to developmental options such as filamentation and meiosis when challenged in G1 phase (Nash et al. 2001; Garí et al. 2001; Wang et al. 2004; Colomina et al. 2008).
Involvement of the phosphorylation of Whi3 in these above-mentioned cellular functions has not been addressed yet. To this end, I identified PKA as the Whi3 kinase, and examined the roles of phosphorylation of Whi3 by PKA in multiple cellular events using the Whi3-S568D and Whi3-S568A mutations. In this chapter, I described that PKA acted as a negative regulator of Whi3. Further, I demonstrated that the phosphorylation of Ser-568 in Whi3 by PKA contributed to cell-cycle control and cell fate determination (Fig. 7).

The cAMP-PKA pathway has been implicated in various cellular processes such as cell-cycle progression, stress response, differentiation, aging, and autophagy (for review, see Matsumoto et al., 1995; Thevelein et al., 1999; Santangelo et al., 2006). Although several PKA substrates have been identified, the biological activities of these proteins are not sufficient to explain the global effect of PKA on numerous cellular events. In this chapter, I showed that the S568D mutation, like the \( \Delta \text{whi3} \) one, accelerated the G\(_1\)/S transition (Fig. 4). In addition, the Whi3-S568D mutation led to defective invasive growth and sporulation as clearly as the \( \Delta \text{whi3} \) mutation (Fig. 5). These findings suggest that the phosphorylation of Whi3 at S568 by PKA played a critical role in the decision of the commitment to the cell-division cycle or to the alternative developmental fates, such as meiosis and invasive growth. The PKA mediated regulation of the Whi3 function has an essential role in the mechanism governing the cell fate decision, a mechanism that inhibits diverse development fate, allowing yeast cells to adapt extracellular change. Under a rich-medium condition, the inhibition of Whi3 function would suppress diverse developmental fates, thus allowing the cells to maintain growth appropriately. Conversely, in poor medium condition, the activation of Whi3 would promote diverse developmental fates for survival. Altogether, it is proposed that the PKA signaling pathway is coordinated to control multicellular processes by regulating Whi3.

Consistent with my results, it was reported that PKA is important for the regulation of the G\(_1\) length in response to glucose (Hall et al., 1998). Conversely, sudden change from a poor carbon source to glucose leads to G\(_1\) delay by the activation of PKA to maintain critical cell size (Baroni et al., 1994; Tokiwa et al., 1994). This discrepancy may lie in the different effects on G\(_1\) cell-cycle progression depending on the signaling pathways and/or the way of experiments. Thus, it suggests that Whi3 is not a major target of PKA in response to a sudden change of nutrition. As shown here, the isolation and
characterization of a direct target of PKA can facilitates the elucidation of the role of PKA in the cell-cycle control.

PKA seems to act as a positive regulator of cell-size control (for review, see Matsumoto et al., 1995; Thevelein et al., 1999; Santangelo et al., 2006). In contrast, my results suggest that PKA acts as a negative regulator of cell-size control through the regulation of Whi3. As nutrients affected the cell size independent of Whi3 (data not shown), Whi3 function by PKA may not be important for the regulation of the nutrient dependent of cell-size control. Further investigation is required for the elucidation of the physiological roles of Whi3 regulated by PKA in the control of cell size.

What is the physiological role of Whi3 function controlled by PKA? Upon exposure of the Δhog1 cells to hyper-osmotic stress, the cells exhibited a severe growth defect, which was suppressed by the sgh4/Whi3 mutation (Fig. 1). Moreover, our recent study revealed that sgh4/whi3 mutation suppressed osmo-sensitivity of Δhog1 cells in a G1 cyclin dependent manner. Further, it was indicated that Hog1 was involved in quick transcriptional activation of the PKA/TPK1 gene, which inactivates the inhibitory function of Whi3 on G1 cyclin, in response to osmotic stress (Mizunuma et al., 2013b). Thus, it is suggested that the activation of PKA mediated by Hog1 pathway with succeeding Whi3 inhibition is important for restart/recovery from G1 delay under high-osmolarity conditions. Alternatively, it is possible that Hog1 and Whi3 antagonize the regulation of restart from G1 arrest caused by osmotic stress. Further analysis would be important for clarification of the molecular mechanism underlying these processes.

Because sgh4/whi3 was originally identified as a suppressor mutation of the Ca2+-sensitive phenotypes of Δhog1 cells, it is indicated that Whi3 is also involved in Ca2+ signaling. I consider the possible relationship between Whi3 and the Ca2+ signaling pathway in CONCLUDING REMARKS.

What is the mechanism for the down-regulation of Whi3 by PKA? The RNA-recognition motif (RRM) of Whi3 would bind the CLN3 mRNA and restrict Cln3 synthesis in the ER (Nash et al. 2001; Gari et al. 2001; Wang et al. 2004; Colomina et al. 2008). Here I showed that PKA phosphorylated the Ser-568 situated within this RRM, thus inhibiting Whi3 function. Therefore, this phosphorylation might have interfered its interaction with CLN3 mRNA, thus escaping the retention mechanism. Indeed, the phosphomimetic Whi3-S568D mutant and the hyperphosphorylated form of Whi3 in the Δbcyl strain showed reduced its interaction with CLN3 mRNA.
I have shown that the *WHI3-S568D* allele behaved like the *whi3* null mutation in all *in vivo* assays described here. However, significant effects of the Whi3-S568A mutation were not observed, except for cell cycle progression. These results suggest that phosphorylation by PKA and/or other kinase(s) at some additional site(s) also contributes to down-regulation of Whi3 function.

From the screening for kinases responsible for Whi3 phosphorylation by using the 119-kinase collection, four kinases (Tpk1, Tpk2, Tpk3, and Ptk2) were identified as Whi3 kinases. Of these, the Ptk2 kinase is involved in the regulation of ion transport and enhances spermine uptake (Longtine *et al.* 1998; Zhu *et al.* 2000). These results indicate that Whi3 is phosphorylated not only by PKA but also by another kinase(s) including Ptk2. Thus, Whi3 would be regulated in multiple ways. Further analysis will be needed for clarification of the physiological roles of phosphorylated Whi3 underlying these processes.

The identification of the phosphorylation site of Whi3 enables direct analysis of Whi3-dependent cyclin expression/cell cycle progression. My data suggest that phosphorylation of Whi3 by PKA played an important role as a direct modulator of a cell fate determination in response to external signals. Since Whi3 homologues are present in some organisms (Nash *et al.* 2001), it will be of great interest to study whether a similar mechanism operates in higher eukaryotes.
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Figure 1  The deletion of the *WHI3* gene suppresses Ca$^{2+}$- and osmo-sensitivity of $\Delta$*hog1* cells.

Effect of the deletion of the *WHI3* gene on the growth of the $\Delta$*hog1* mutant strain on solid medium. WT, $\Delta$*hog1*, $sgh4$, $\Delta$*whi3*, $\Delta$*hog1 sgh4*, $\Delta$*whi3*, and $\Delta$*hog1 $\Delta$whi3* cells were spotted on YPD solid medium containing 300 mM CaCl$_2$, 0.6 M Sorbitol or 0.4 M KCl after which the plates were incubated at 28°C for 3 days.
Figure 2  Whi3 is phosphorylated by PKA in vitro and in vivo.

(A) Protein phosphatase treatment of Whi3-HA. Whole-cell extracts of WT cells expressing Whi3-HA were incubated with (+) or without (-) l-phosphatase (P\textsubscript{Pase}) or the phosphatase inhibitor. After separation by SDS-PAGE, Whi3-HA was detected by immunoblotting. Arrows indicate the phosphorylated (*) and dephosphorylated (none) forms of Whi3-HA protein. ** indicate the non-specific band. (B) Recombinant MBP-Whi3 or MBP (control) was incubated with GST-Tpk1, GST-Tpk2, or GST-Tpk3 in the presence of [\gamma\textsuperscript{-32}P]ATP (\gamma\textsuperscript{-32}P). Phosphorylated Whi3 was separated by SDS-PAGE and detected by autoradiography (upper panel). MBP and MBP-Whi3 proteins were purified from \textit{E. coli} BL40 and stained with Coomassie Brilliant Blue (CBB; lower panel). (C) Schematic representation of full-length Whi3. The location of the RRM and the position of Ser-568 are indicated. (D) MBP-RRM, MBP-RRM-S568A, or MBP (control) was incubated with GST-Tpk1 in the presence of [\gamma\textsuperscript{-32}P]ATP. Phosphorylated RRM was separated by SDS-PAGE and detected by autoradiography (upper panel). Phosphorylation levels were quantified by phosphorimaging, and relative values normalized to MBP-RRM are indicated below each band. Means ± S.E. of three independent experiments are shown. The difference was statistically significant (middle panel). **, \( p < 0.005 \) by \( t \) test. MBP, MBP-RRM, and MBP-RRM-S568A proteins were purified from \textit{E. coli} BL40 and stained with Coomassie Brilliant Blue (lower panel). (E) Mobility shift of Whi3-HA or Whi3 S568A-HA from the wild-type (WT) and \( \Delta \text{bcy1} \) cells. WT and \( \Delta \text{bcy1} \) cells expressing Whi3-HA or Whi3 S568A-HA were grown in YPD medium to mid-log phase. Proteins of whole-cell extracts were separated by SDS-PAGE and detected by immunoblotting with anti-HA (for Whi3-HA and Whi3 S568A-HA) and anti-PSTAIRE (for Cdc28 as a loading control) antibodies. Arrows indicate phosphorylated Whi3-HA proteins with different mobilities. (F) Protein phosphatase treatment of Whi3-HA. Whole cell extracts of WT and \( \Delta \text{bcy1} \) cells expressing Whi3-HA (lanes 2–7) were incubated with (+) or without (-) \( \lambda \)-phosphatase or a phosphatase inhibitor. \textit{Lane 1} is a negative control lacking the HA tag on Whi3. After separation by SDS-PAGE, Whi3-HA was detected by immunoblotting. Arrows indicate the hyperphosphorylated (**), phosphorylated (*), and dephosphorylated forms of Whi3-HA protein. A nonspecific band is indicated (***).
Figure 3  PKA controls cell size by phosphorylating Whi3.
(A) Whi3-HA, Δwhi3, Whi3-S568A-HA, and Whi3-S568D-HA cells were grown in YPD medium. Relative cell size was determined by measuring forward angle light scattering by using a FACSCalibur (Becton Dickinson). Each histogram was obtained from 2 x 10⁴ cells.
Figure 4  Phosphorylation state of Whi3 at its Ser568 by PKA affects the timing of CLN2 transcription and cell-cycle progression.

(A) Effect of Whi3-S568A or Whi3-S568D mutations on cell-cycle progression. The Whi3-HA, Whi3-S568A-HA, Whi3-S568D-HA, and Δwhi3 strains were synchronized with α-factor and released into YPD. (B) Effect of Whi3-S568A or Whi3-S568D mutations on CLN2 transcription. Northern blot analysis was performed for CLN2 and ACT1 (control) mRNA levels in the cells taken periodically after having been synchronized with α-factor in YPD. (C) Changes in the relative intensity of the CLN2 and ACT1 mRNA bands. The amount of the CLN2 mRNA in B was normalized by that of the ACT1 mRNA. For each strain, the maximum value for the first cell cycle was referred to as 1.
Figure 5  Phosphorylation state of Whi3-Ser568 is important for the growth regulation to switch to or not to switch to meiosis and invasive cell growth.

(A) Effect of Whi3-S568A or Whi3-S568D mutation on the invasive growth of the haploid ΣMLY41 strain on YPD plates. WT, Whi3-S568A-HA, Whi3-S568D-HA, Awhi3, Acln3, Acln3 Whi3-S568A-HA, Acln3 Whi3-S568D-HA or Acln3 Awhi3 cells were streaked onto a YPD plate and cultured at 28ºC for 2 days. (B) Efficiency of spore formation after 48 h of cultivation of the diploid strains grown in sporulation medium. Data represent average of 3 independent experiments. Error bars show the mean ± SEM.
Figure 6  Phosphorylation of Whi3 by PKA inhibits binding of Whi3 to \textit{CLN3} mRNA \textit{in vivo}.

Extracts of cells expressing Whi3-HA, Whi3-S568D-HA, Whi3-S568A-HA, \textit{Δbcy1 Whi3-HA}, or \textit{Δbcy1 Whi3-S568A-HA} were immunoprecipitated as described under “Experimental Procedures.” Each sample was separated by SDS-PAGE and detected by immunoblotting with anti-HA antibodies. RNA was extracted from cell extracts (Total) and immunoprecipitates (IP) and used as template for RT-qPCR. The relative intensity of the \textit{CLN3} mRNA was normalized to that of \textit{ACT1} mRNA (Total). Values relative to the \textit{CLN3} mRNA level of Whi3-HA (+) are indicated below each bar. Data represent the means ± S.E. of three independent experiments.
Figure 7  Model for the role of PKA in regulating multiple cellular functions by phosphorylating Whi3 at Ser-568.
CHAPTER II

Implication of Ca$^{2+}$ in the Regulation of Replicative Life Span of Budding Yeast
2.1 ABSTRACT

In eukaryotic cells, the calcium ion (Ca$^{2+}$) is involved in the regulation of various cellular functions as a signal molecule. The $zds1\Delta$ strain of the *Saccharomyces cerevisiae* shows the sensitivity to Ca$^{2+}$ and the Ca$^{2+}$-induced G$_2$ cell cycle arrest, and polarized bud growth, due to the activation of the cellular Ca$^{2+}$-signaling pathways mediated by the calcineurin and Mpk1 MAP kinase pathway. Calcineurin is a highly conserved Ca$^{2+}$/calmodulin dependent protein phosphatase, and plays key roles in the regulation of diverse biological processes in organisms from yeast to humans. To uncover the growth control regulated by the Ca$^{2+}$-signaling pathways, we screened for suppressor mutants of the Ca$^{2+}$-sensitive phenotypes of $zds1\Delta$ cells (scz mutants). Of these mutants, we identified mutant allele of *SIR3*, involved in the regulation of life span, as *scz14*. However, the relationship between the Ca$^{2+}$-signaling and the aging of yeast is obscure. Therefore, I focused on the relationship between Ca$^{2+}$ signaling and life span in yeast. Here I show that Ca$^{2+}$ affected the replicative life span (RLS), which is defined by the number of mother cell divisions before senescence. I found that cells grown in the medium supplemented with high Ca$^{2+}$ showed decreased RLS relative to normal medium. It suggests that increased intracellular Ca$^{2+}$ levels, and consequent activation of the calcineurin caused a reduction in RLS. Indeed, the deletion of $ZDS1$ gene, resulted in the increase in cellular Ca$^{2+}$ level, and the constitutively activation of calcineurin led to a shortened RLS. Further, the shortened RLS of $zds1\Delta$ cells was suppressed by the calcineurin deletion. Moreover, I demonstrate that the strain lacking *CNB1* gene, encodes the regulatory subunit of calcineurin, had the deceased RLS, suggesting that the basal activity of calcineurin is important for maintain normal life span in yeast. Thus, these results indicate that Ca$^{2+}$ homeostasis/ Ca$^{2+}$ signaling are important for regulation of longevity in budding yeast.

2.2 INTRODUCTION

The calcium ion (Ca$^{2+}$) is a highly universal intracellular signal that regulates many different cellular processes such as cell proliferation, muscle contraction, development, fertilization, motility, memory, and apoptosis (for review, see Berridge *et al*., 2000; Berridge *et al*., 2003). Moreover, it was reported that $\alpha$-klotho, which was originally identified as a putative age-suppressing gene in mice, is involved in the regulatory
system of Ca\textsuperscript{2+} metabolism, suggesting a relationship between Ca\textsuperscript{2+} and aging (Imura et al., 2007).

In the budding yeast *Saccharomyces cerevisiae*, the Ca\textsuperscript{2+}/calmodulin-dependent protein phosphatase calcineurin has been implicated in stress-induced gene expression, cation homeostasis, cell cycle regulation, cell wall synthesis, and recovery from the mating pheromone induced G\textsubscript{1} arrest (Aramburu et al., 2000; Cyert et al., 2001; Miyakawa et al., 2007). Recent reports showed that inactivating calcineurin slows aging in *Caenorhabditis elegans* (Dwivedi et al., 2009; Mair et al., 2007). However, the relationship between Ca\textsuperscript{2+} signaling and aging in yeast has not been known.

Most of the identified regulatory factors of aging are evolutionally conserved from yeast to higher eukaryotes, suggesting the implication of common mechanisms of aging in diverse organisms. For instance, dietary restriction is known as the common way to extend the life span and to increase stress resistance in organism ranging from yeast to mammals (for review, see Kenyon, 2010; Fontana et al., 2010). In budding yeast, reducing the concentration of glucose in the culture medium extends yeast life span (Lin et al., 2000). This extension from dietary restriction is accounted for the activation of the silent information regulator (Sir) protein Sir2, a central determinant of yeast life span (Lin et al., 2000; Lin et al., 2002). Sir2 is a family of NAD\textsuperscript{+}-dependent protein deacetylases (Imai et al., 2000; Finkel et al., 2009) required for the transcriptional silencing at mating type loci *HML* and *HMR* (Rine et al., 1987), at telomeres (Aparicio et al., 1991; Gottschling et al., 1990), and at the rDNA locus *RDN1* (Bryk et al., 1997; Fritze et al., 1997; Smith et al., 1997). It has been showed that deletion of either *SIR2*, *SIR3*, or *SIR4* results in a shortened life span, whereas overexpression of its product increase life span, suggesting that the Sir complex functions to promote longevity (Kaeberlein et al., 1999; Kennedy et al., 1997).

Mutations in *ZDS1* (zillion different screens) gene have been found by a wide variety of genetic screens (Bi et al., 1996; Ma et al., 1996; Yu et al., 1996). The Zds1 function reported include chromatin silencing, the establishment of cell polarity, cell cycle progression, and diverse other processes (Bi et al., 1996; Ma et al., 1996; Yu et al., 1996; Roy et al., 2000; Mizunuma et al., 1998; Griffoen et al., 2003; Zanelli et al., 2005; Rossio et al., 2011; Calabria et al., 2012; Rossio et al., 2013). Moreover, Zds1 is also implicated the regulation of life span (Roy et al., 2000). Although the important
roles of Zds1 (and its homolog Zds2) in various cellular events have been indicated, its biochemical function is not completely known.

Previously, we showed that ZDS1 null mutant cells cultivated in the medium containing a high concentration of CaCl₂ were delayed in the G₂ phase and showed polarized bud growth, due to the activation of cellular Ca²⁺ signaling pathways in budding yeast (Nakamura et al., 1996; Mizunuma et al., 1998). Two Ca²⁺ activated pathways, namely, the Mpk1 MAP kinase cascade and the calcineurin pathway, coordinately regulate the G₂/M transition. To identify novel signaling components of the cell cycle and the growth control mediated by Ca²⁺ signaling, we screened for suppressor mutants of the Ca²⁺-sensitive phenotypes of zds1 deletion cells and they had been classified into 14 genetic complementation groups (designated scz1 to scz14 for suppressor of Ca²⁺-induced abnormalities of the Δzds1 strain). Of these mutants, we identified the mutant allele of SIR3 as scz14. In this chapter, I investigated the relationship between Ca²⁺ signaling and replicative life span (RLS) in yeast. I propose that Ca²⁺ homeostasis/Ca²⁺ signaling is required for regulation of RLS.

2.3 MATERIALS AND METHODS

2.3.1 Yeast Strains and Media

Yeast strains used in this study are listed in Table 2. All yeast strains were derivatives of W303. The media used were as described previously (Mizunuma et al., 1998).

2.3.2 Gene disruption and strain construction

The Δsir3, Δpmc1, and Δpmr1 strains on a W303 background were constructed by gene replacement. Genomic DNA was isolated from the Δsir3::kanMX4, Δpmc1::kanMX4, and Δpmr1::kanMX4 strains on a BY4741 background (Invitrogen). The amplified fragment was used to transform a haploid W303 strain.

2.3.3 Yeast Replicative Life Span (RLS)

RLS analyses were determined as described previously (Kennedy et al., 1995). Cells selected randomly were aligned on the life span plate with a micromanipulator. After each cell division, the newly born daughter cells were left there, and the mother cells were removed. Starting with this newly born cells, the daughter cells produced after
each cell division were removed. This procedure was continued until cell division finished every 2-4 hours. The RLSs of these cells were determined by the number of daughters that were generated. All RLS analyses in this chapter were carried out on YPD plates at least 3 times, independently. Statistical significance was determined by performing a log rank test. Average RLSs were taken to be significantly different at $p < 0.05$. Results from a single experiment are shown.

2.3.4 β-Galactosidase Assays

Cells containing pKC201 (Cunningham et al., 1996) were grown to mid log phase at 28°C in SC minus uracil. The cells were harvested and resuspended in fresh YPD medium (pH 5.5). After incubation for an additional 4 hours at 28°C with shaking, the cells were provided for assay of β-Galactosidase activity as described previously (Guarente, 1983). Error bars represent the standard deviation of the mean (SD). Statistical significance between the wild-type and other strains was determined by performing a t-tests.

2.4 RESULTS

2.4.1 The Deletion of the SIR3 Gene Confers Ca$^{2+}$ Resistance to Δzds1 Cells

ΔZDS1 null mutant (Δzds1) yeast cells grown in medium containing a high concentration of CaCl$_2$ (300 mM) were delayed in G$_2$ phase and displayed highly polarized bud growth, due to the activation of the cellular Ca$^{2+}$ signaling pathways (Mizunuma et al., 1998). As shown in Fig. 8A-C, the growth inhibition and the polarized bud growth, but not the G$_2$ delay, of Δzds1 cells due to exogenous CaCl$_2$ were suppressed by an additional mutation scz14. The plasmid that complemented the phenotype of the scz14 mutants was cloned, and it was revealed that it contained the SIR3 gene. Linkage of SIR3 to the SCZ14 locus was confirmed by tetrad analysis (data not shown). In fact, the deletion of the SIR3 gene, similarly as the scz14 mutation, suppressed the growth inhibition and the polarized bud growth, but not the G$_2$ delay, of Δzds1 cells, suggesting that the scz14 mutation is a loss-of-function allele of the SIR3 gene (Fig. 8A-C). For this reason, I used the Δsir3 cells in further experiments.

Why did the Δsir3 mutation suppress only the Ca$^{2+}$-induced hyperpolarized bud growth but not the G$_2$-delay of Δzds1 cells? Because the Ca$^{2+}$ signal induced the
polarized bud growth by elevating the level of G₁-cyclin Cln2 (Mizunuma et al., 2005), I investigated whether the sir3 deletion affected the Cln2 level by performing Western blot analysis of strains with chromosomally integrated construct for HA-tagged Cln2. The protein levels of Cln2 in the presence and absence of exogenous calcium in Δzds1 cells were decreased by the disruption of SIR3 (compare Δzds1 and Δzds1 Δsir3 in Fig. 8D), suggesting that the suppression of the Ca²⁺ sensitive phenotype of the Δzds1 cells by the sir3 deletion was caused by a defect in the elevation of the Cln2 level in response to Ca²⁺. Additionally, similar effect of the sir3 disruption on the Cln2 level was observed on wild-type (WT) background (compare WT and Δsir3 in Fig. 8D). These results indicate that Sir3 is important for the elevation of the Cln2 level in the presence and absence of external Ca²⁺. The Sir3 protein in budding yeast is required for telomere silencing (Aparicio et al., 1991) and also seems to play a role in the life span regulation (Austriaco et al., 1997; Kaeberlein et al., 1999; Roy et al., 2000). Therefore, in this chapter, I focused on a relationship between Ca²⁺-signaling and lifespan.

2.4.2 Increase in External and Intracellular Ca²⁺ Levels Causes Shortened RLS

I first investigated whether the extracellular Ca²⁺ would affect the lifespan. Budding yeast typically divides asymmetrically to give a large mother cell and a smaller daughter cell. Aging in budding yeast is measured as a RLS (replicative life span), i.e., the number of mother cell divisions before senescence (Kennedy et al., 1994; Sinclair et al., 1997; Defossez et al., 2001). Cells grown in the YPD medium supplemented with 100 mM CaCl₂ showed a decreased RLS relative to normal medium (Fig. 9A). To check the specificity of Ca²⁺ on the yeast RLS, I examined the effect of MgCl₂ and KCl on RLS, because some cations (such as Na⁺, Li⁺, Mn²⁺, Co²⁺, and Ni²⁺) but not Mg²⁺ and K⁺ have been shown to affect the Ca²⁺ homeostasis (Nakamura et al., 1993; Farcasanu et al., 1995). As the result, neither MgCl₂ (100 mM) nor KCl (150 mM) altered significantly the RLS of WT cells (Fig. 9A), indicating that external Ca²⁺ reduced RLS.

To investigate the possibility that extracellular Ca²⁺ shortens RLS by activating intracellular Ca²⁺ signaling, I measured the RLS of Δzds1 cells, in which Ca²⁺-signaling pathways might be activated by increase in internal Ca²⁺ level (Mizunuma et al., 1998). As expected, the mean RLS of Δzds1 cells was shorter than that of WT cells under the normal condition (Fig. 9B).

To examine whether the zds1 deletion affected the activation of Ca²⁺-signaling
pathways under the experimental condition, I investigated calcineurin activity in vivo by using a reporter assay system (Cunningham et al., 1996). In response to the elevation of the cellular Ca\(^{2+}\) level, the transcription factor Crz1 binds to calcineurin-dependent response element (CDRE) in the promoter and activates several stress-responsive target genes (such as VCX1, PMC1, PMR1, and PMR2A). I used the CDRE-driven LacZ reporter gene (Cunningham et al., 1996) and analyzed the calcineurin activity in the WT and zds1Δ cells under normal growth condition (YPD) by β-galactosidase assay. The β-galactosidase activity in WT cells indicated the basal level of this CDRE-driven LacZ reporter system, because the activity in WT cells was comparable to that in the calcineurin-deleted cells (Δcnb1) (Fig. 9C). As expected, β-galactosidase activity in the Δzds1 cells increased significantly by 3-fold compared to that in WT cells (Fig. 9C), and this increase was indeed dependent on calcineurin (Fig. 9C, compare between Δzds1 and Δzds1 Δcnb1). These results suggested that the increase in cellular Ca\(^{2+}\) level in the Δzds1 cells causes the shortened RLS by activating Ca\(^{2+}\)-signaling pathway(s).

2.4.3 Increase in Cellular Ca\(^{2+}\) Level by PMR1 deletion Shortened RLS

We previously identified two genes, PMR1 and PMC1 as the multi-copy suppressors of the Ca\(^{2+}\)-sensitive phenotypes of zds1Δ cells (Yokoyama et al., 2006). The genes, PMR1 and PMC1, encode Golgi and vacuolar Ca\(^{2+}\)-ATPases ion pump, respectively (Rudolph et al., 1989; Antebi et al., 1992; Cunningham et al., 1994a; Cunningham et al., 1994b). Both Pmr1 and Pmc1 transport Ca\(^{2+}\) from the cytosol into each intracellular compartment. In addition, it has been reported that PMR1 deletion causes the alternations of intracellular Ca\(^{2+}\) homeostasis, including an increased rate of cellular Ca\(^{2+}\) uptake from the extracellular environment and an enhanced sensitivity to high extracellular Ca\(^{2+}\) levels (Halachmi et al., 1996; Kellermayer et al., 2003).

To clarify whether the maintenance of calcium homeostasis is important for longevity, I observed the RLS of the Δpmr1 and Δpmlc1 cells. The Δpmr1 cells but not the Δpmlc1 cells had a severe reduction in mean RLS of ~ 64% (Fig. 9D), indicating that Pmr1-mediated Ca\(^{2+}\) transport into Golgi, but not Pmc1-mediated one into vacuole, is important for longevity under normal condition. Further, to investigate the role of these Ca\(^{2+}\)-ATPases in cellular Ca\(^{2+}\) homeostasis, I measured calcineurin activity in these cells by using the CDRE-driven LacZ reporter system (Cunningham et al., 1996). Consistent with the result of RLS, β-galactosidase activity in the Δpmr1 cells, but not in
the Δpme1, significantly increased by 4-fold, compared to the basal level in the WT cells (Fig. 9C). These results indicate that Pmr1-mediated Ca\(^{2+}\) sequestration to Golgi plays an important role in regulation of RLS through intracellular Ca\(^{2+}\) homeostasis.

### 2.4.4 Hyper-Activation of Calcineurin Shortens the RLS

To further confirm the above suggestion that activated Ca\(^{2+}\) signaling pathways decreases the RLS, I investigated whether over-expression of the constitutively activated calcineurin (CMP2ΔC, C-terminal auto-inhibitory domain-truncated catalytic subunit) promotes aging and shortens the RLS. Indeed, the WT cells expressing the activated calcineurin (YEp24-CMP2ΔC) had a reduced RLS compared to the WT cells carrying the empty plasmid (YEp24) (Fig. 10A). This result indicated that activation of Ca\(^{2+}\)/calcineurin-signaling pathway(s) shortens RLS.

### 2.4.5 Calcineurin-Deletion Overcomes the Shortened RLS of Δzds1 Cells

We previously reported that calcineurin and Zds1 are implicated in an antagonistic regulation of cell growth and morphogenesis in the presence of high Ca\(^{2+}\) (Mizunuma et al., 1998). Because disruption of ZDS1 resulted in about 20% decrease in the mean RLS with hyper-activation of calcineurin (Fig. 9B), I speculated that calcineurin deletion overcomes the shortened lifespan in Δzds1 cells. As expected, Δzds1 Δcnb1 double deletion cells displayed a longer RLS compared to Δzds1 single deletion cells (Fig. 10B). Interestingly, the mean RLS of Δzds1 Δcnb1 cells was comparable to that of the WT cells. These results indicate that calcineurin and Zds1 play an antagonistic role in the regulation of RLS and the activated calcineurin promotes ageing in the Δzds1 cells.

### 2.4.6 Calcineurin and Sir3 Act Redundantly in the Regulation of RLS

I next examined the effect of the calcineurin deletion (Δcnb1) on the RLS. Surprisingly, the calcineurin deletion per se on the WT background shortened RLS under normal growth condition (Fig. 10B and C), suggesting that the basal activity of calcineurin is required for longevity under this condition.

In the Sir3-mediated RLS regulation, the Mpk1 MAPK cascade was identified as the pathway that phosphorylates Sir3, which results in shorter RLS (Ray et al., 2003). We and other previously showed that calcineurin and Mpk1 act redundantly in cellular events (Garrett et al., 1995; Mizunuma et al., 1998; Nakamura et al., 1996). To
investigate whether calcineurin is related to the Sir3-mediated RLS regulation, I examined the RLS of ∆cnb1 ∆sir3 double deletion cells. As previously reported (Kaeberlein et al., 1999), deletion of SIR3 leads to a 22% decrease in the mean RLS (Fig. 10C). The mean RLS of ∆cnb1 ∆sir3 double deletion cells was shortened more compared with that of each single deletion mutant (Fig. 10C). These results indicated that calcineurin and Sir3 act redundantly in regulation of RLS as parallel pathways.

2.5 DISCUSSION

In this chapter II, I showed that calcineurin has an important role in the regulation of yeast life span. Hyper activation of calcineurin-signaling shortened RLS (Fig. 10A). Indeed, the shortened RLS of ∆zds1 cells, in which calcineurin is activated by the increase in cellular Ca^{2+} level, was suppressed by the calcineurin deletion (Fig. 10B). These results indicate that the constitutive activation of calcineurin would be toxic to yeast in the long term, although it is required for the resistance to several stresses, including ionic stress (Na^{+}, Li^{+}, and Mn^{2+}), high pH, and the presence of cell wall-disrupting compounds (calcofluor white and Congo red). Surprisingly, disruption of calcineurin gene also promoted the aging in the WT cells, suggesting that the calcineurin activity in basal level suppresses aging under normal growth condition. However, in the multicellular organism Caenorhabditis elegans, a calcineurin-deficient mutant displayed an extended lifespan (Dwivedi et al., 2009; Mair et al., 2011). Moreover, I observed that all scz mutation caused the shortened RLS in yeast (unpublished data). Thus, it is possible that an optimal level of calcineurin activity/expression would be required for longevity, and Ca^{2+} signaling pathways are important for longevity in the unicellular organism such as budding yeast.

How do calcineurin and Zds1 act in the regulation of RLS? My results suggested that calcineurin and Zds1 play an antagonistic role in the regulation of RLS. Previously, we reported that the growth inhibition, the polarized bud growth, and the G_{2} delay by high Ca^{2+} in ∆zds1 cells were suppressed by the calcineurin deletion mutations (Mizunuma et al., 1998). Moreover, calcineurin activity was enhanced in the ∆zds1 cells (Fig. 9C). Therefore, it is possible that Zds1 may be required for the longevity in the negative regulation of calcineurin. Further investigation is required for the nature of the Zds1 functions.
Why did the Δpmr1 cells show a severe reduction in mean RLS compared to other cells (Δzds1, Δcnb1, and Δsir3)? The Pmr1 protein plays an important role in both protein sorting and proton transport (Dürr et al., 1998; Yadav et al., 2007). Although none of the gene that is related to the secretary pathway has been identified yet as the RLS regulator, it has been reported that some proton ATPase mutants, such as the VMA genes encoding vacuolar H1-ATPases, reduced RLS (Kaeberlein et al., 2005). These results suggest that the severe RLS reduction of Δpmr1 cells was derived not only from altered Ca2+ homeostasis but also from defect of proton transport. As the double deletion between calcineurin and VMA gene displays synthetic lethality (Tanida et al., 1995; Nakamura et al., 1996), the shortened RLS of the calcineurin deletion might be caused at least in part by the defect in control of the intracellular pH. Although a functional interaction between secretion and RLS has not been shown, I have not ruled out this possibility.

Previously, we showed that calcineurin leads to the Cln2 up-regulation by a mechanism that is mediated by degradation of Yap1 transcription factor (Yokoyama et al., 2006). In this chapter, I showed that Sir3 was required for the up-regulation of the Cln2 level (Fig. 8D). In the regulation of RLS, a functional link between Sir3 and Cln2 has not yet been identified. However, my results suggest that the optimum levels of Cln2 might be important for the longevity in yeast. My findings also suggest that Ca2+ homeostasis is necessary for longevity in yeast.

It will be interesting to determine whether altered Ca2+ homeostasis influences the life span of higher eukaryotes such as human, and whether the corresponding pathways are a part of a conserved network involved in the control of biological aging.
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Figure 8  The deletion of SIR3 gene suppresses various phenotypes of the Δzds1 strain.

(A) Effect of the deletion of SIR3 gene on the growth of the Δzds1 mutant strain on solid medium. WT, Δzds1, Δscz14, Δsir3, Δzds1 Δscz14, and Δzds1 Δsir3 cells were spotted on YPD solid medium containing 300 mM CaCl₂, after which the plates were incubated at 25°C for 3 days. (B, C) Cell morphology (DIC: Differential Interference Contrast) after 6 h of incubation with 100 mM CaCl₂ at 25°C (B). Flow cytometry analysis (FACS) of PI-stained cells of various strains after 6 h of incubation with 100 mM CaCl₂ at 25°C (1C: 1 DNA copy or 2C: 2 DNA copies) (C). (D) Cln2-HA and Cdc28 were detected by Western blotting using early log-phase growing cells of WT, Δzds1, Δsir3, and Δzds1 Δsir3 strain were suspended in YPD containing 100 mM CaCl₂, incubated for 6 h, and then used for Western blotting. Cln2-HA and Cdc28 were detected by immunoblotting with anti-HA or anti-PSTAIRE antibody, respectively.
Figure 9  An increase in Ca\textsuperscript{2+} levels led to reduced RLS.

(A) RLS analysis of WT cells grown on YPD (control), YPD plus 100 mM CaCl\textsubscript{2}, YPD plus 100 mM MgCl\textsubscript{2}, and YPD plus 150 mM KCl at 25\degree C. Mean RLS in terms of numbers of cell divisions: control, 27.4 (n = 52); 100 mM CaCl\textsubscript{2}, 22.5 (n = 36); 100 mM MgCl\textsubscript{2}, 29.8 (n = 52); 150 mM KCl, 28.3 (n = 52). (B) RLS analysis of WT and Δzds1 mutant cells grown on YPD at 25\degree C. Mean RLSs: WT, 24.3 (n = 43); Δzds1, 19.6 (n = 44). (C) Expression of the CDRE-dependent reporter gene in WT, Δzds1, Δzds1 Δcnb1, Δpmc1, Δpmr1, and Δcnb1 mutant cells. *P<0.001 (D) RLS analysis of WT, Δpmc1, and Δpmr1 mutant cells grown on YPD at 25\degree C. Mean RLSs: WT, 27.5 (n = 47); Δpmc1, 26.3 (n = 44); Δpmr1, 9.4 (n = 49).
Figure 10  Effect of over-expression and deletion of calcineurin on RLS.

(A) RLS analysis of the cells over-expressing constitutively active form of calcineurin. WT cells transformed with empty plasmid (YEp24) or YEp24-CMP2ΔC plasmid grown on YPD at 25ºC were examined. Mean RLSs: YEp24, 25.5 (n = 40); YEp24-CMP2ΔC, 21.9 (n = 39). (B) RLS analysis of WT, Δzds1, Δcnb1, and Δzds1 Δcnb1 mutant cells grown on YPD at 25ºC. Mean RLSs: WT, 26.9 (n =44); Δzds1, 21.8 (n = 44); Δcnb1, 23.5 (n = 44); Δzds1 Δcnb1, 27.6 (n = 43). (C) RLS analysis of WT, Δcnb1, Δsir3, and Δcnb1 Δsir3 mutant cells grown on YPD at 25ºC. Mean RLSs: WT, 25.2 (n = 18); Δcnb1, 19.7 (n = 33); Δsir3, 19.6 (n = 20); Δcnb1 Δsir3, 15.0 (n = 36).
CONCLUDING REMARKS

The eukaryotic cells have developed various signaling pathways in order to cope with the change of the environment. The mechanism of signal transduction is evolutionally conserved from unicellular microorganisms such as yeast to multicellular organisms including insects, plants, and mammals. The researches on intracellular signaling pathways have disclosed diverse biological phenomena such as immunity, development, cancer and aging at the molecular level (for review, see Silverman et al., 2001; Pires-daSilva and Sommer, 2003; Show and Cantley, 2006; Kenyon, 2010). The cause of various diseases including cancer has been explained as aberrations in the molecules of the signaling pathways, and medical biology is accomplishing an epochal advance (for review, see Sebolt-Leopold and English, 2006). However, the physiological roles of the signaling pathway remained largely unknown, and the interaction of various signaling pathways still largely remain to be investigated.

Aging is a universal biological phenomenon, which is observed in every organism from microorganism to human. It is a complex process accompanied by accumulation of various molecular, cellular, organ damages, leading to diseases and death. Despite the complexity of aging process, it is suggested that the aging process, as well as many other biological processes, is subject to regulation by signaling pathways and transcription factors (for review, see Kenyon, 2005; Kenyon, 2010; Fontana et al., 2010). Although many reports have shown that dietary and genetic alternations can increase life span of various model organisms, the molecular mechanisms that determine life span are poorly understood.

The budding yeast *Saccharomyces cerevisiae* has emerged as a versatile and robust model system of eukaryotic cells. Genetic and molecular analyses are simpler and easier to perform in yeast than in multicellular organisms. Then, it serves as powerful analysis tools for understanding the molecular mechanisms of highly complex signaling pathways in higher eukaryotes such as human (for review, see Forsburg, 2005; Botstein and Fink, 2011). Different signaling pathways and transcription factors coordinate the expression of stress response genes. Moreover, it has been used as a useful model for aging research, leading to the identification of new longevity genes and pathways in higher eukaryotes (for review, see Sinclair et al., 1998; Bitterman et al., 2003; Kaeberlein, 2010). How much of what we learn about signaling pathways in yeast is relevant to human has become an important question. Although some aspects of
signaling pathways in yeast are specific to this organism, the evidences so far suggest that many of the most important features have been evolutionarily conserved in mammals.

In chapter I, I showed that the RAS/cAMP-dependent protein kinase (PKA) functioned as the Whi3 kinase. Whi3 was phosphorylated by PKA and that the phosphorylation of Ser-568 in Whi3 by PKA had an inhibitory role in the Whi3 function. Phosphorylation of Whi3 by PKA resulted in its decreased interaction with CLN3 mRNA and was required for the promotion of the G1/S progression. Further, I demonstrated that the phospho-mimetic S568D mutation of Whi3 prevented the developmental options including sporulation and invasive growth. Thus, PKA modulated the function of Whi3 by phosphorylation, and implicating PKA-mediated modulation of Whi3 in multiple cellular events (Mizunuma et al., 2013a). Because Whi3 homologs are present in some organisms (Nash et al., 2001), it will be interesting to study whether a similar mechanism is operated in human.

In chapter II, I showed that Ca2+ affected the replicative life span (RLS) of yeast. I described that increased external and intracellular Ca2+ levels caused decreased RLS. Consistently, the deletion of ZDS1 gene, which showed the increased cellular Ca2+ level, and the activation of calcineurin led to a shortened RLS. Further, the shortened RLS of Δzds1 cells was suppressed by the calcineurin deletion. Moreover, I demonstrated that the strain lacking CNBI gene had the deceased RLS, suggesting that the basal activity of calcineurin is essential for longevity in yeast. Thus, our results indicated that Ca2+ homeostasis/ Ca2+ signaling are required to regulate longevity in budding yeast. We also reported that other SCZ genes (a suppressor mutation of the Ca2+-sensitive phenotypes of Δzds1 cells) were involved in the regulation of yeast life span (Tsubakiyama et al., 2011). Since the calcium ion (Ca2+) is a universal second messenger in various organisms, it will be very interesting to investigate whether the collapse of Ca2+ homeostasis affects the life span of higher eukaryotes.

In this thesis, I clarified the role of Whi3 phosphorylated by PKA in the cell fate determination, and the relationship between Ca2+ homeostasis/ Ca2+ signaling and life span regulation for the first time in yeast. Because we identified the mutant allele of WHI3 as sgh4, which was a suppressor mutation of the Ca2+-sensitive phenotypes of Δhog1 cells, it is expected that Whi3 is also involved in Ca2+ signaling. How dose Whi3 function in Ca2+ signaling pathways? Our recent study indicated that Whi3 acted as a
negative regulator for restart from G₁ arrest under osmotic stress (Mizunuma et al., 2013a). Moreover, it was revealed that cells showed transient G₁ arrest under high Ca²⁺ conditions, and that high Ca²⁺ caused the delay of CLN2 mRNA accumulation, which was abolished by the *whi3* mutation (unpublished data). These results indicate the possibility that Whi3 function mediated by PKA may play a role in the regulation of the restart from G₁ arrest under high Ca²⁺ conditions as well as osmotic stress. We previously reported that the Ca²⁺ signaling pathways, through the activation of calcineurin, and Hog1 pathway, which plays a central role in response to hyperosmotic stress, are implicated in an antagonistic regulation of cell growth (Shitamukai et al., 2004). Moreover, our results indicated that PKA was quickly activated by the osmotic signal in a Hog1-dependent manner (Mizunuma et al., 2013b). Based upon these findings suggest that the Ca²⁺ signaling pathways and PKA may antagonize one another in the regulation of cell growth. Further investigation is required for the nature of the Whi3 functions.

Many evidences have been shown the relationship between signaling pathways regulating cell proliferation and aging of various organisms (for review, see Kenyon, 2005; Kenyon, 2010; Fontana et al., 2010). Indeed, it was reported that mutation in the PKA pathway affected life span (Fabrizio et al., 2001; Lin et al., 2002; Tong et al., 2007). Since it was suggested that mutation of the PKA pathways have shown to extended life span in yeast (Fabrizio et al., 2001; Lin et al., 2002), Whi3 may function downstream of PKA to promote longevity.

Whi3 regulates the various physiological roles such as cell size, normal mating response, filamentous growth and meiosis. Interestingly, recent report showed that yeast cells are able to form a memory through the super-assembly of Whi3 (Caudron et al., 2013). Yeast cells unproductively exposed to pheromone enter a pheromone refractory state, and this state is memorized by mother cells, not inherited by daughter cells. This ‘memory’ is encoded by super-assembly and inactivation of Whi3. Super-assembly of Whi3 may act as cellular memory devices to encode previous environmental conditions, and this memory system may be regulated by PKA-Whi3 pathway. It will be interesting to investigate whether a similar memory mechanism is also thought to exist in the nerve cells of higher organisms, and whether this memory system is linked to longevity.

There are close relations between signaling pathways of cell proliferation and many diseases such as cancer, diabetes, autoimmune diseases, and neurological diseases.
Mutations in signaling pathways that control cell proliferation cause various disorders. Ras1/2, which are upstream regulator of yeast adenylate cyclase (activated Ras1/2 indirectly activate PKA through the stimulation of cAMP), are a homolog of the mammalian RAS protein. The mammalian RAS protein was originally identified as a gene that has the potential to cause cancer (called as oncogene). In tumor cells, Ras gene are often mutated or expressed at high levels (for reviews, see Malumbres et al., 2003). Not only Ras-PKA signaling pathway but also the Ca^{2+} signaling pathways have been implicated in varied disorders. Calcineurin, which has a central role in Ca^{2+} signaling pathways, is the target of the immunosuppressant drugs cyclosporin A and FK506 and several viral immune modulators. In addition, its dysfunction is implicated in Down’s syndrome, diabetes, and cardiac hypertrophy (for reviews, see Carafoli, 2002; Berridge et al., 2003; Puzianowska-Kuznicka and Kuznicki, 2009).

Moreover, susceptibility to a wide variety of diseases increases with age. Importantly, long-lived mutants are resistant to these age-related diseases including Huntington’s diseases, sarcopenia, heart failure, and cancer (Morley et al., 2002; Herndon et al., 2002; Ramsey et al., 2002; Wessells et al., 2004). Hence, future studies in yeast and other model organisms to identify novel signaling pathways functioning in cell growth and life span regulation are likely to open up therapeutic opportunities for many diseases of human.
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RELATED PUBLICATIONS

(1) cAMP/PKA regulates multiple aspects of cellular events by phosphorylating Whi3 cell-cycle regulator in budding yeast.
Masaki Mizunuma, Ryohei Tsubakiyama, Takafumi Ogawa, Atsunori Shitamukai, Yoshifumi Kobayashi, Tomomi Inai, Kazunori Kume and Dai Hirata
The Journal of Biological Chemistry, 288 (15), 10558-10566 (2013)

(2) Implication of Ca\(^{2+}\) in the regulation of replicative life span of budding yeast.
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(1) cAMP/PKA regulates multiple aspects of cellular events by phosphorylating Whi3 cell-cycle regulator in budding yeast.
Masaki Mizunuma, Ryoei Tsubakiyama, Takafumi Ogawa, Atsunori Shitamukai, Yoshifumi Kobayashi, Tomomi Inai, Kazunori Kume and Dai Hirata
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(2) Implication of Ca²⁺ in the regulation of replicative life span of budding yeast.
Ryohei Tsubakiyama, Masaki Mizunuma, Anri Gengyo, Josuke Yamamoto, Kazunori Kume, Tokichi Miyakawa and Dai Hirata
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The Start/G1 phase in the cell cycle is an important period during which cells determine their developmental fate, onset of mitotic progression, or the switch to developmental stages in response to both external and internal signals. In the budding yeast *Saccharomyces cerevisiae*, Whi3, a negative regulator of the G1 cyclins, has been identified as a positive regulator of cell size control and is involved in the regulation of Start. However, the regulatory pathway of Whi3 governing the response to multiple signals remains largely unknown. Here, we show that Whi3 is phosphorylated by the Ras/cAMP-dependent protein kinase (PKA) and that phosphorylation of Ser-568 in Whi3 by PKA plays an inhibitory role in Whi3 function. Phosphorylation of Whi3 by PKA plays an important role as a direct modulator of cell fate decision in response to external signals.

**Background:** Whi3 is known as a negative regulator of the G1 cyclin.

**Results:** The phosphorylation of Ser-568 in Whi3 by PKA plays an inhibitory role in Whi3 function.

**Conclusion:** Phosphorylation of Whi3 by PKA plays an important role as a direct modulator of cell fate decision in response to external signals.

**Significance:** A new aspect of the interface between the cell cycle and cell fate has been discovered.

Whi3 in budding yeast was originally identified as a positive regulator of cell size control and is involved in the regulation of Start (3). The best characterized function of Whi3 is its ability to inhibit Cln3 function in the G1 phase by binding to *CLN3* mRNA, thereby inhibiting the Cln3-Cdc28-mediated activation of two transcription factors, SBF (Swi4-Swi6) and MBF (Mbp1 and Swi4), which drive the expression of the G1 cyclin *CLN1* and *CLN2* mRNAs and lead to a delay in activation of Start of the cell cycle (4–7). In addition, Whi3 function is required for developmental options such as invasive growth and meiosis (4). However, little is known about the upstream regulator(s) of Whi3.

The Ras/cAMP-dependent protein kinase (PKA) pathway in yeast has been implicated in numerous cellular processes, including carbon storage, stress response, growth, differentiation, and life span (for reviews, see Refs. 8–10). The molecular mechanism by which yeast cells sense and respond to the specific stimuli generated by PKA has been studied extensively. One of the most prominent roles of PKA signaling is known to be the regulation of the critical cell size required for Start in response to nutrient conditions (for reviews, see Refs. 8–10). PKA has been implicated in cell size control by nutritional conditions such as nutrient levels, and decreased PKA signaling results in decreased cell size, whereas hyperactive PKA signaling leads to increased cell size (11–13), indicating that PKA is a positive regulator of cell size control.

In addition, the PKA pathway appears to play a crucial role in the connection between the availability of nutrient signals and G1/S transition. It has long been thought that the G1 length of cells growing rapidly in rich medium is shorter than that of cells growing slowly in poor medium and that cells in rich medium also have higher PKA activity than those in poor medium. Genetic evidence supports this idea that PKA increases the expression of the Cln3-Cdc28 kinase complex to promote passage through Start in rich medium (14). On the other hand, other data indicate that PKA delays Start during the shift from poor to rich medium (12, 13). These results indicate that PKA plays an important role in Start as a positive or negative regu-
lator depending on the nutrient conditions/shifts. Thus, the involvement of PKA in the regulation of Start remains enigmatic. Despite the obvious importance of the PKA signaling pathway, only a few substrates/targets of PKA in the control of cell growth have been identified (9, 10).

In this study, we identify PKA as the Whi3 kinase. We show that the phosphorylation of Ser-568 in Whi3 by PKA plays an inhibitory role in Whi3 function. This mechanism is essential for the acceleration of G1/S progression. Furthermore, we demonstrate that the phosphomimetic S568D mutation of Whi3 prevents sporulation and invasive growth. On the basis of these findings, we propose that the phosphorylation of Whi3 by PKA is involved in multiple cellular events, including cell cycle control and developmental fate in response to environmental stimuli.

EXPERIMENTAL PROCEDURES

Yeast Strains and Media—The yeast strains used in this study were as follows: W303-1A (MATa trp1-1 leu2-3 ade2-1 his3-11 can1-100), SHI301 (MATa Δwhi3::kanMX6), SH1294 (MATa WHI3-3HA::kanMX6), YMM501 (MATa Δbcycl1::URA3 WHI3-3HA::kanMX6), YMM502 (MATa WHI3-S568A-3HA::kanMX6), YMM504 (MATa Δbcycl1::URA3 WHI3-S568A-3HA::kanMX6), YMM505 (MATa WHI3-S568D-3HA::kanMX6), YRT73 (MATa Δcln3::HIS3 WHI3-3HA::kanMX6), YRT76 (MATa Δcln3::HIS3 WHI3-S568A-3HA::kanMX6), YRT77 (MATa Δcln3::HIS3 WHI3-S568D-3HA::kanMX6), YRT74 (MATa Δcln3::HIS3 Δwhi3::kanMX6), YTO1 (MATa HIS3 GAL1-WHI3-3HA::kanMX6), YTO2 (MATa HIS3 GAL1-WHI3-S568D-3HA::kanMX6), YMM507 (MATa/α WHI3-S568A::kanMX6), YMM508 (MATa/α WHI3-S568A-3HA::kanMX6), YMM509 (MATa/α WHI3-S568D-3HA::kanMX6), YMM510 (MATa/α Δwhi3::kanMX6/Δwhi3::kanMX6), YRT91 (MATa/α Δcln3::HIS3 WHI3-3HA::kanMX6/Δcln3::HIS3 WHI3-3HA::kanMX6), YRT94 (MATa/α Δcln3::HIS3 WHI3-S568A-3HA::kanMX6/Δcln3::HIS3 WHI3-S568A-3HA::kanMX6), YRT93 (MATa/α Δcln3::HIS3 WHI3-S568D-3HA::kanMX6/Δcln3::HIS3 WHI3-S568D-3HA::kanMX6), and YRT92 (MATa/α Δcln3::HIS3 Δwhi3::kanMX6/Δcln3::HIS3 Δwhi3::kanMX6), all derivatives of strain W303-1A. Also used were the following derivatives of the Σ strain: MLY41a (MATa ura3-52), YMM515 (WHI3-S568A-3HA::kanMX6 in MLY41a), YMM516 (WHI3-S568D-3HA::kanMX6 in MLY41a), YMM17 (Δwhi3::kanMX6 in MLY41a), YRT85 (Δcln3::URA3 ura3-52 in MLY41a), YMR78T (Δcln3::URA3 WHI3-S568A-3HA::kanMX6 in MLY41a), YRT88 (Δcln3::URA3 WHI3-S568D-3HA::kanMX6 in MLY41a), and YRT86 (Δcln3::URA3 Δwhi3::kanMX6 in MLY41a). The media used were as described previously (15).

Site-directed Mutagenesis and Construction of Plasmids—The pMBP-WHI3 plasmid harboring the fusion gene for the maltose-binding protein (MBP)4–Whi3 conjugate protein was constructed as follows. The WHI3 gene was amplified by PCR, digested with BamHI and Sall, and then cloned into the BamHI- and Sall-digested pMAL-C2 vector (provided by A. Kikuchi).

The pMBP-WHI3-S568A plasmid harboring the fusion gene for the mutant MBP-Whi3-S568A conjugate protein was constructed using a QuikChange™ XL site-directed mutagenesis kit (Stratagene) and the pMBP-WHI3 plasmid as a PCR template. The pMBP-RRM plasmid harboring the fusion gene for the MBP-Whi3 RNA recognition motif (RRM) conjugate protein was constructed as follows. The RRM domain was amplified by PCR, digested with BamHI and Sall, and then cloned into the BamHI- and Sall-digested pMAL-C2 vector. The pMBP-RRM-S568A plasmid harboring the fusion gene for the mutant MBP-RRM-S568A conjugate protein was constructed using the QuikChange™ XL site-directed mutagenesis kit and the pMBP-RRM plasmid as a PCR template. The pWhi3-S568A-3HA plasmid was constructed as follows. First, the BamHI-Sall fragment of the pMBP-WHI3-S568A plasmid was cloned into BamHI- and Sall-digested pUC119 to construct pWhi3-S568A. Next, the WHI3 gene containing a 3-HP epitope tag was amplified from genomic DNA of the WHI3-3HA::kanMX6 strain (provided by Dr. M. Aldea) by PCR and cloned into the pT7Blue vector (Novagen) to construct pT-Whi3-5T-3HA. Finally, the Apal-Sphl fragment of the pT-Whi3-5T-3HA plasmid was cloned into the Apal- and Sphl-digested pWhi3-S568A plasmid. The pWhi3-S568D-3HA plasmid was constructed similarly using the pWhi3-S568A-3HA plasmid as the PCR template. The mutations were confirmed by DNA sequencing.5

Gene Disruption and Strain Construction—The Δwhi3 strain was constructed by gene replacement. Genomic DNA was isolated from the whi3::kanMX4 strain on a BY4741 background (Invitrogen). The PCR-amplified fragments of whi3::kanMX4 were used to transform the W303-1A and Σ MLY41a strains (16). Deletion of the genomic CLN3 gene (CLN3 plasmids provided by I. Yamashita) and the BCY1 gene was performed using a disruption plasmid.

The strains with chromosomally integrated genes for WHI3-S568A and WHI3-S568D with a 3-HP epitope at their C termini were constructed as follows. The pWhi3-S568A-3HA and pWhi3-S568D-3HA plasmids were digested with NspV (within the WHI3 locus) and used to transform the appropriate strains. Insertion of these fragments into the original WHI3 locus was confirmed by DNA sequencing and Western blot analysis.

In Vitro Phosphatase Assay—Cell extracts (200 µg of total protein) were incubated with 400 units of λ-phosphatase (New England Biolabs) in 50 µl of λ-phosphatase buffer and 1 mM MnCl2 with or without phosphatase inhibitors (50 mM NaF and 5 mM sodium orthovanadate) for 60 min at 30 °C. After the phosphatase treatment, 4X sample buffer for SDS-PAGE was added, and the mixture was boiled for 10 min. Whi3-HA was detected by immunoblotting.

In Vitro Protein Kinase Assay—The procedure used for the in vitro kinase assay was carried out as described previously (17). Expression of the MBP-Whi3 fusion protein in Escherichia coli BL21 was induced by the addition of 0.1 mM isopropyl β-D-thiogalactopyranoside to the culture medium. MBP-Whi3 protein was affinity-purified using amylose resin beads (New Eng-
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land Biolabs) according to the manufacturer’s instructions. Protein kinases from a collection of 119 yeast protein kinases fused to glutathione S-transferase (kindly provided by M. Snyder) (17) were overexpressed in yeast and affinity-purified using glutathione-Sepharose 4B beads (Amersham Biosciences). The MBP-Whi3 and GST-fused protein kinases on the beads were mixed in 50 μl of kinase buffer containing 0.5 μCi of [γ-32P]ATP, 100 μM ATP, and 10 mM MgCl2 and incubated at 30 °C for 30 min. After the beads had been washed with the kinase buffer, the proteins were eluted by boiling the beads in SDS sample buffer for 5 min. The eluted proteins were resolved by SDS-PAGE and detected by autoradiography.

Invasive Growth Assay—Yeast cells (ΔMLY41a, provided by J. Heitman) were streaked onto a yeast extract/peptone/dextrose (YPD) plate and allowed to grow at 28 °C for 2 days. The plate was photographed before and after rinsing with a gentle stream of water to remove all of the cells from the agar surface.

Sporulation Conditions—For induction of sporulation, cells grown in YPD medium were shifted to 1% yeast extract, 2% peptone, and 2% potassium acetate; grown for at least three generations at 28 °C; and harvested at a density of 3–5 × 107 cells/ml. The cells were washed twice with sporulation medium (1% potassium acetate), resuspended at 1.5 × 107 cells/ml in the same medium, and further incubated at 28 °C. The formation of ascii was assessed by phase-contrast microscopy.

RNA Binding, Immunoprecipitation, and mRNA Detection—Exponentially growing cells (4 × 107) were disrupted with glass beads in 200 μl of extraction buffer (50 mM Tris-HCl (pH 7.5), 150 mM KCl, and 2 mM MgCl2) containing 40 units/μl RNasin (Qiagen), phosphatase inhibitors (10 mM NaF and 1 mM sodium orthovanadate), 0.1% Nonidet P-40, 1 mM DTT, 1 mM PMSF, 10 μg/ml aprotinin, and protease inhibitor mixture (α-Complete, Roche Applied Science). Extracts were cleared by centrifugation for 15 min at 14,000 × g. Anti-HA monoclonal antibody (HA.11, Berkeley Antibody Co.) and protein A-agarose beads were added to the cleared extracts, followed by incubation for 8 h at 4 °C. The beads were then washed four times with wash buffer (50 mM Tris-HCl (pH 7.5) containing 150 mM KCl and 2 mM MgCl2) and subsequently eluted for 10 min at 65 °C in 50 mM Tris-HCl (pH 8.0) containing 100 mM NaCl, 10 mM EDTA, and 1% SDS. Eluted samples were extracted according to standard protocols (Qiagen). RT-PCR was performed with 2 μl of RNA as template using the One-Step SYBR PrimeScript RT-PCR Kit II (TaKaRa) and the conditions suggested by the manufacturer.

RESULTS

Identification of PKA as the Whi3 Kinase—Whi3 was identified previously as a negative regulator of the G1 cyclin for cell size control (3) and shown to play an important role in regulating Cln3 activity in G1 (4). Despite its linkage to multiple cellular events, including cell cycle progression and developmental fate, the upstream regulator(s) of Whi3 has not still been fully identified. Protein phosphorylation plays a pivotal role in the regulation of many cellular functions. As the phosphorylation of Whi3 had not been analyzed yet, we examined whether Whi3 could be phosphorylated in vivo. We detected slowly migrating bands of Whi3-HA on an SDS-polyacrylamide gel (Fig. 1A, lane 2). The phosphatase-treated Whi3-HA protein migrated faster...
than the untreated protein (Fig. 1A, compare lanes 2 and 3), indicating that Whi3 had been phosphorylated under normal growth conditions.

To identify the protein kinase(s) responsible for the phosphorylation of Whi3, we screened for the kinase that is capable of phosphorylating the recombinant Whi3 protein fused to MBP in vitro, using [γ-32P]ATP as the phosphate donor, from a collection of 119 potential protein kinases consisting of those previously characterized and predicted in the yeast genome database. From this screening, we found that Tpk1, one of the three isoforms of the cAMP-dependent protein kinase (PKA), exhibits the highest kinase activity for MBP-Whi3 but shows no activity toward MBP (Fig. 1B, lanes 1 and 2). The two other isoforms of PKA, i.e. Tpk2 and Tpk3, exhibit moderate kinase activity in this assay (Fig. 1B, lanes 3 and 4).

To identify the site(s) of Whi3 phosphorylated by PKA, we searched for the consensus sequence for PKA-mediated phosphorylation (R(R/K)X(S/T)) (18) in Whi3 and found only one consensus sequence (Ser-568) situated within the RRM of Whi3 (Fig. 1C). To examine whether Ser-568 is phosphorylated in vitro, we generated a RRM of Whi3 that contained amino acids 539–621 and then constructed non-phosphorylatable mutant RRM-S568A recombinant protein by site-directed mutagenesis, after which we performed an in vitro kinase assay. The phosphorylation level of MBP-RRM-S568A was statistically significantly reduced compared with that of MBP-RRM but to a modest extent (Fig. 1D), suggesting that Ser-568 of Whi3 is one of the phosphorylation sites mediated by PKA in vitro.

To confirm that PKA phosphorylates Whi3 in vivo, we examined whether the phosphorylation level of Whi3 would be elevated by deletion of the PKA negative regulator BCY1 gene, in which case, PKA becomes constitutively active. As expected, in the bcy1 cells, the phosphorylation level of Whi3-HA increased (Fig. 1E, lanes 1 and 2). The phosphatase-treated Whi3-HA protein in bcy1-deleted cells migrated faster than the untreated protein (Fig. 1F, compare lanes 3 and 5), confirming that Whi3 had been phosphorylated by PKA in vivo. To examine whether Ser-568 is a phosphorylation site of Whi3 in vivo, we constructed the non-phosphorylatable mutation Whi3-S568A by site-directed mutagenesis and compared the phosphorylation levels of Whi3-HA and the Whi3-S568A-HA mutant protein in both WT and bcy1-deleted cells (Fig. 1E). In the WT cells, the phosphorylation level of the Whi3-S568A-HA mutant protein was indistinguishable from that of the Whi3-HA protein (Fig. 1E, lanes 1 and 3), suggesting that an additional phosphorylation site(s) of Whi3 recognized by PKA might exist in Whi3. In contrast, in the bcy1 cells, the major mobility shift in the Whi3-HA protein was not observed in the Whi3-S568A-HA mutant protein (Fig. 1E, lanes 2 and 4). These results indicate that Ser-568 of Whi3 is the site phosphorylated by PKA in vivo.

**Phosphomimetic Whi3-S568D Mutation Decreases Cell Size**—The above results indicate that Ser-568 of Whi3 is one of the major phosphorylation sites recognized by PKA in vivo. Thus, we focused on the role of this phosphorylation of Whi3 in multiple cellular events. As the WHI3 gene was originally identified as a positive regulator of cell size control (3), we first investigated whether the phosphorylation state of Ser-568 in Whi3 participates in this control. Wild-type (Whi3-HA) and Whi3-S568A-HA cells growing exponentially in normal YPD medium were similar in size. In contrast, the size of phosphomimetic Whi3-S568D-HA mutant cells, like that of the deletion mutant (Δwhi3), was smaller compared with the WT cells (Fig. 2A). These results indicate that the phosphorylation of Whi3 by...
PKA causes a decrease in cell size by down-regulating Whi3 function. PKA activation leads to larger cell size in response to nutrient conditions (13). We examined whether Whi3 is involved in this cell size control, using glucose and ethanol as carbon sources. As reported previously (13), the cell size of wild-type cells growing in glucose medium was larger than that in ethanol medium (Fig. 2B). The cell size of all whi3 mutant strains (Δwhi3, Whi3-S568D-HA, and Whi3-S568A-HA) was also similarly responsive to these carbon sources as the wild-type cells. These results suggest that Whi3 is not a major target of PKA in terms of cell size control in response to a nutrient signal.

We noted that the effect of a whi3 mutation (Whi3-S568D and Whi3-S568A) on the expression levels of these proteins was comparable (Fig. 3A). Because phosphomimetic Whi3-S568D mutant cells seemed to behave like the Δwhi3 cells, we asked whether overexpression of Whi3-S568D would produce a phenotype or not. WHI3 expressed from the GAL1 promoter is lethal and causes an increase in cell size (3). For this reason, we examined the effect of GAL-WHI3-S568D-HA on cell size (Fig. 3B).
Phosphorylation of Ser-568 in Whi3 Is Important for Cell Fate Determination—Whi3 is important for restraining Cln3 function in the G₁ phase, leading to sporulation or invasive growth (4). Coincidentally, PKA has been shown to be required for the growth regulation of the switch to sporulation and filamentous growth (9). Thus, we examined the effects of the Whi3 mutations on sporulation and invasive growth. The haploid Whi3-S568D-HA mutant, but not the Whi3-S568A-HA mutant, was defective in invasive growth (Fig. 5A). Furthermore, the homozygous diploid Whi3-S568D-HA mutant, but not the Whi3-S568A-HA mutant, failed to sporulate (Fig. 5B). As it has been reported that defects in both sporulation and invasive growth of Δwhi3 cells are alleviated by the loss of CLN3 (4), we examined whether these deficiencies in the Whi3-S568D-HA mutant are caused by promotion of the G₁/S transition through the up-regulation of Cln3. As expected, both defects in the Whi3-S568D-HA mutant were reversed by the CLN3 deletion (Fig. 5A and B). These results indicate that the PKA-mediated phosphorylation of Ser-568 in Whi3 is essential for cell fate determination in the G₁ phase.

Phosphorylation of Whi3 by PKA Leads to Its Decreased Interaction with CLN3 mRNA—All of the above results are consistent with the idea that PKA-mediated phosphorylation of Whi3 at Ser-568 inhibits Whi3 function. How did PKA down-regulate it? Whi3 negatively regulates CLN1, CLN2, and CLN3 mRNA levels by binding to these mRNAs, most efficiently to CLN3 mRNA (4). Thus, we speculated that the phosphomimetic Whi3-S568D mutant and the hyperphosphorylated form of Whi3 in the ∆bcy1 strain would show decreased interaction with CLN3 mRNA. To examine this possibility, we carried out Whi3 immunoprecipitation followed by RT-PCR analysis to measure Whi3 association with CLN3 mRNA. As expected, both the phosphomimetic (Whi3-S568D-HA) and hyperphosphorylated (Δbcy1 Whi3-HA) forms of Whi3 showed reduced interaction with CLN3 mRNA in this assay (Fig. 6A). Conversely, the Whi3-S568A mutant displayed increased interaction with CLN3 mRNA (Fig. 6A). Moreover, the increase in the ability of Whi3-S568A to bind to CLN3 mRNA was still observed in the ∆bcy1 mutation background (see FIGURE 4. Phosphorylation state of Whi3 at Ser-568 by PKA affects the timing of CLN2 transcription and cell cycle progression. A, effect of the Whi3-S568A and Whi3-S568D mutations on cell cycle progression. The Whi3-HA, Whi3-S568A-HA, Whi3-S568D-HA, and ∆whi3 strains were synchronized with α-factor and released into YPD medium. B, effect of the Whi3-S568A and Whi3-S568D mutations on CLN2 transcription. Northern blot analysis was performed for CLN2 and ACT1 (control) mRNA levels in the cells taken periodically after synchronization with α-factor in YPD medium. C, changes in the relative density of the CLN2 and ACT1 mRNA bands. The amount of the CLN2 mRNA in ∆whi3 was normalized to that of the ACT1 mRNA. For each strain, the maximum value for the first cell cycle is referred to as 1.
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Δbcy1 Whi3-S568A mutant), although the binding ability was slightly reduced by the Δbcy1 mutation. This result indicates that the Whi3-S568A mutation is epistatic to the Δbcy1 mutation and further supports our model that Ser-568 of Whi3 is a major regulatory site phosphorylated by PKA for down-regulating the ability of Whi3 to bind to CLN3 mRNA. Altogether, these observations suggest that PKA promotes G1/S progression by phosphorylating Whi3, which in turn causes reduced interaction with CLN3 mRNA.

Whi3-associated mRNAs are enriched in clusters of the tetranucleotide GCAU, and mutation of these clusters in the CLN3 mRNA causes a reduction in its association with Whi3 (7). Thus, cells harboring mutated clusters (CLN3mGCAU) in their CLN3 mRNA due to mutations in the 14 GCAT sites in the CLN3 gene show a decrease in cell size similar to Δwhi3 cells (7). If Whi3-S568A binds through GCAU sites in the CLN3 mRNA, Whi3-S568A would not be able to bind to the CLN3mGCAU mRNA, and so Whi3-S568A cells harboring CLN3mGCAU should show a decrease in cell size. We therefore tested whether Whi3 Ser-568 contributes to the interaction with GCAU sites in the CLN3 mRNA by monitoring cell size. As reported previously (7), the size of the Δcln3 Whi3-HA cells harboring the CLN3mGCAU mRNA allele was smaller than that of those harboring CLN3, as in the case of the Δcln3 Whi3-HA cells (Fig. 6B). In contrast, the CLN3mGCAU mRNA had no significant effect on the size of the Δcln3 Whi3-S568D-HA or Δcln3 Δwhi3 cells (Fig. 6B). These results support the idea that phosphorylation of Ser-568 in Whi3 by PKA inhibits the interaction of Whi3 with GCAU clusters in the CLN3 mRNA.

DISCUSSION

Whi3 function is important not only for control of the G1/S transition but also for the switch to developmental options such as invasive growth and meiosis (3, 4, 6, 7). None of the above studies addressed the role of the phosphorylation of Whi3 in such cellular functions. To this end, we identified PKA as the Whi3 kinase and examined the consequences of phosphorylation of Ser-568 in Whi3 by PKA in multiple cellular events using the Whi3-S568D and Whi3-S568A mutations. In this study, we showed that PKA acts as a negative regulator of Whi3. Furthermore, we demonstrated that the phosphorylation of Ser-568 in Whi3 by PKA contributes to cell cycle control and cell fate determination (Fig. 7).

The PKA pathway has been implicated in numerous cellular processes such as cell cycle progression, stress response, and differentiation (for reviews, see Refs. 9 and 10). Although several PKA substrates have been described, the biological activities of these proteins are not sufficient to explain the global effect of PKA on multicellular events. In this study, we showed that like the Δwhi3 mutation, the S568D mutation accelerated

FIGURE 5. Phosphorylation state of Whi3 Ser-568 is important for growth regulation to switch to or not to switch to meiosis or invasive cell growth. A, effect of the Whi3-S568A or Whi3-S568D mutation on the invasive growth of the haploid ΔMY41a strain on YPD plates. WT, Whi3-S568A-HA, Whi3-S568D-HA, Δwhi3, Δcln3, Δcln3 Whi3-S568A-HA, Δcln3 Whi3-S568D-HA, or Δcln3 Δwhi3 cells were streaked onto YPD plates and cultured at 28 °C for 2 days. Efficiency of spore formation after 48 h of cultivation of the diploid strains grown in sporulation medium. Data represent the means ± S.E. of three independent experiments.

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the G1/S transition (Fig. 4). In addition, we demonstrated that the S568D mutation was defective in invasive growth and sporulation as clearly as the \textit{whi3} mutation (Fig. 5). These findings suggest that the phosphorylation of Whi3 at Ser-568 by PKA plays a critical role in the decision for commitment to the cell division cycle or to alternative developmental fates such as meiosis and invasive growth. The regulation of Whi3 function by PKA appears to play an essential role in the mechanism governing the cell fate decision. In rich medium, the inhibition of Whi3 function would suppress diverse developmental fates, thus allowing the cells to maintain growth appropriately. Conversely, in poor medium, the activation of Whi3 would promote diverse developmental fates for survival. We propose that the PKA signaling pathway is coordinated to control multicellular processes by regulating Whi3.

Consistent with our results, PKA was reported to be required for acceleration of the passage through G1 in response to glucose (14). Conversely, a sudden change from a poor carbon source to glucose leads to a G1 delay by activation of PKA to maintain a critical cell size (12, 13). This discrepancy may lie in the different effects on G1 cell cycle progression depending on the signaling pathways and/or the type of experiments. Thus, Whi3 may not be a major target of PKA with regard to G1 cell cycle progression when cells respond to a sudden change in nutrition. As shown here, elucidation of the role of PKA in cell cycle control can be facilitated by the isolation and characterization of a direct target of PKA.

PKA seems to act as a positive regulator of cell size (for reviews, see Refs. 8–10). In contrast, our results suggest that PKA appears to act as a negative regulator of cell size through the regulation of Whi3. As nutrients affected the cell size independently of Whi3 (Fig. 2), the physiological roles of Whi3 regulated by PKA in the control of cell size remain unclear. What is the mechanism for the down-regulation of Whi3 by PKA? The RRM of Whi3 would bind the CLN3 mRNA and restrict Cln3 synthesis in the endoplasmic reticulum (3, 4, 6, 7). Here, we showed that PKA phosphorylated Ser-568 of this RRM, thus inhibiting Whi3 function. Therefore, this phosphorylation might have disturbed the association between Whi3 and CLN3 mRNA, thus escaping the retention mechanism. Indeed, the phosphomimetic Whi3-S568D mutants and the hyperphosphorylated form of Whi3 in the \textit{whi3} strain showed reduced interaction with CLN3 mRNA (Fig. 6A).

We have shown that the \textit{WHI3-S568D} allele behaved like the \textit{whi3} null mutation in all \textit{in vivo} assays described here. However, prominent effects of the \textit{Whi3-S568A} mutation were not observed, except for cell cycle progression. These results suggest that phosphorylation by PKA and/or other kinase(s) at some additional site(s) also contributes to down-regulation of Whi3 function.
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Among the 119 kinase tested, we identified four kinases (Tpk1, Tpk2, Tpk3, and Ptk2) as Whi3 kinases. Of these, the Ptk2 kinase is involved in the regulation of ion transport and enhances spermine uptake (19, 20). These results indicate that Whi3 is phosphorylated not only by PKA but also by another kinase(s). Thus, Whi3 would be regulated in multiple ways. Further analysis will be needed for clarification of the physiological roles underlying these processes.

The identification of the phosphorylation site of Whi3 enabled us to directly analyze Whi3-dependent cyclin expression/cell cycle progression. Our data suggest that phosphorylation of Whi3 by PKA plays an important role as a direct modulator of a cell fate decision in response to external signals. Because Whi3 homologs are present in some organisms (3), it will be of great interest to study whether a similar mechanism operates in higher eukaryotes.

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Implication of Ca\(^{2+}\) in the Regulation of Replicative Life Span of Budding Yeast*\(^{\dagger}\)

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In budding yeast, the Ca\(^{2+}\)-triggered signaling pathways are used to regulate a wide variety of cellular processes. Calcineurin, a highly conserved Ca\(^{2+}\)/calmodulin-dependent protein phosphatase, plays key roles in the regulation of diverse biological processes in organisms ranging from yeast to humans. We isolated a mutant of the SIR3 gene, implicated in the regulation of life span, as a suppressor of the Ca\(^{2+}\) sensitivity of zds1\(^{Δ}\) cells in the budding yeast *Saccharomyces cerevisiae*. Therefore, we investigated a relationship between Ca\(^{2+}\) signaling and life span in yeast. Here we show that Ca\(^{2+}\) affected the replicative life span (RLS) of yeast. Increased external and intracellular Ca\(^{2+}\) levels caused a reduction in their RLS. Consistently, the increase in calcineurin activity by either the zds1 deletion or the constitutively activated calcineurin reduced RLS. Indeed, the shortened RLS of zds1\(^{Δ}\) cells was suppressed by the calcineurin deletion. Further, the calcineurin deletion per se promoted aging without impairing the gene silencing typically observed in short-lived *sir* mutants, indicating that calcineurin plays an important role in a regulation of RLS even under normal growth condition. Thus, our results indicate that Ca\(^{2+}\) homeostasis/Ca\(^{2+}\) signaling are required to regulate longevity in budding yeast.

The calcium ion (Ca\(^{2+}\)) is a universal second messenger important in the regulation of diverse biological processes such as cell proliferation, muscle contraction, fertilization, development, motility, memory, and apoptosis (1). In addition, it was reported that α-Klotho, which was originally identified in short-lived mutant mice, is a major player in the regulatory system of Ca\(^{2+}\) homeostasis, suggesting a link between Ca\(^{2+}\) and aging (2).

In budding yeast, the Ca\(^{2+}\)/calmodulin-dependent protein phosphatase calcineurin has been implicated in stress-induced gene expression, ion homeostasis, cell cycle regulation, and maintenance of viability after exposure to the mating pheromone (1, 3, 4). A recent report demonstrated that a calcineurin-deficient mutant of *Caenorhabditis elegans* displays an extended life span (5, 6). However, the relationship between Ca\(^{2+}\) and aging in yeast is not known.

Most of the identified regulatory factors of aging are evolutionally conserved, suggesting the implication of common processes/pathways in regulation of life span and aging in diverse organisms. For example, calorie restriction is a dietary regimen that is known to extend the life span and to increase stress resistance in organisms from yeast to mammals (7, 8). In yeast, a decrease in the glucose concentration of the culture medium extends the life span (9). This extension requires the activation of the silent information regulator (Sir) protein Sir2, a central determinant of yeast life span (9, 10). Sir2 is an NAD-dependent histone deacetylase (11–13) required for the chromatin silencing in mating-type loci HML and HMR (14), telomeres (15, 16), and the ribosomal DNA (rDNA) locus RDN1 (17–19). Because deletion of either SIR2, SIR3, or SIR4 results in a shortened life span (20), the Sir complex functions to promote longevity in the wild-type cells.

Mutations in ZDS1 (zillion different screens) gene have been isolated in numerous genetic screenings for its ability to suppress the phenotypes caused by defects in various genes when it is overexpressed on a high-copy vector (21–23). The Zds1 functions reported include chromatin silencing, the establishment of cell polarity, cell cycle progression, and numerous other multiple processes (21–29). Moreover, Zds1 is also implicated in the regulation of aging (24). Although Zds1 (and its homolog Zds2) appears to be important in a wide range of cellular events, its biochemical function is not completely known.

We showed previously that ZDS1 null mutant budding yeast cells cultured in medium containing a high concentration of CaCl\(_2\) were delayed in the G\(_2\)/M phase and displayed polarized bud growth because of the activation of cellular Ca\(^{2+}\) signaling pathways (25). Two Ca\(^{2+}\)-activated pathways, namely, the Mpk1 MAP kinase cascade and the calcineurin pathway, coordinately regulate the G\(_2\)/M cell cycle transition. To search for novel signaling components that mediate or affect the Ca\(^{2+}\)-dependent regulation of cell growth and morphogenesis, we isolated mutant strains and classified them into 14 genetic complementation groups (designated *scz1* to *scz14* for suppressor of Ca\(^{2+}\)-induced abnormalities of the zds1\(^{Δ}\) strain). One of the identified mutants, *scz4*, was a mutant allele of *sir3*. In this study, we investigated a relationship between Ca\(^{2+}\) signaling and replicative life

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2 The abbreviations used are: rDNA, ribosomal DNA; RLS, replicative life span; YPD, yeast peptone dextrose.
span (RLS) in yeast. Our results indicate that Ca\(^{2+}\) homeostasis/Ca\(^{2+}\) signaling are required for regulation of RLS.

**EXPERIMENTAL PROCEDURES**

**Strains—**Yeast strains DHT22-1b (MATa trp1 leu2 ade2 ura3 his3 can1–100), YAT1 (MATa zds1Δ::TRPl), YJ10 (MATa zds1Δ::TRPl sir3Δ::kanMX4), YJ9 (MATa sir3Δ::kanMX4), YGA13 (MATa pmc1Δ::kanMX4), YGA14 (MATa pmr1Δ::kanMX4), DHT14 (MATa cnb1Δ::HIS3), YJ12 (MATa cnb1Δ::HIS3 sir3Δ::kanMX4), DMY2798 (MATa leu2::mUra3-LEU2), YSS7 (zds1Δ::kanMX4 in DMY2798), YGA4 (cnb1Δ::kanMX4 in DMY2798), YSS8 (zir3Δ::kanMX4 in DMY2798), YGA9 (cnb1Δ::HIS3 sir3Δ::kanMX4 in DMY2798), DMY2827 (sir2Δ::kanMX4 in DMY2798), DMY2800 (MATa NTS2::mUra3-LEU2), YSS5 (zds1Δ::kanMX4 in DMY2800), YGA6 (cnb1Δ::kanMX4 in DMY2800), YSS10 (sir3Δ::kanMX4 in DMY2800), YGA11 (cnb1Δ::HIS3 sir3Δ::kanMX4 in DMY2800), DMY2831 (sir2Δ::kanMX4 in DMY2800), DMY2804 (MATa NTS1::mUra3-LEU2), YSS4 (zds1Δ::kanMX4 in DMY2804), YGA5 (cnb1Δ::kanMX4 in DMY2804), YSS9 (sir3Δ::kanMX4 in DMY2804), YGA10 (cnb1Δ::HIS3 sir3Δ::kanMX4 in DMY2804), DMY2835 (sir2Δ::kanMX4 in DMY2804), YGA2895 (MATa ura3::TRPl), YS51 (zir3Δ::kanMX4 in DMY2895), YGA2 (cnb1Δ::kanMX4 in DMY2895), YS6 (zir3Δ::kanMX4 in DMY2895), YGA7 (cnb1Δ::HIS3 sir3Δ::kanMX4 in DMY2895), DMY2841 (sir2Δ::kanMX4 in DMY2895), DMY2896 (MATa TEL VII::Ura3), YSS2 (zds1Δ::kanMX4 in DMY2896), YGA3 (cnb1Δ::kanMX4 in DMY2896), YSS7 (sir3Δ::kanMX4 in DMY2896), YGA8 (cnb1Δ::HIS3 sir3Δ::kanMX4 in DMY2896), and DMY2839 (sir2Δ::kanMX4 in DMY2896) were the derivatives of strain DHT22-1b (MATa trp1 leu2 ade2 ura3 his3 can1–100). The oligonucleotide primer sequences were used to transform a strain. The amplified fragment was obtained from yeast. Our results indicate that Ca\(^{2+}\) homeostasis/Ca\(^{2+}\) signaling are required for regulation of RLS.

**Role of Ca\(^{2+}\) in Yeast Aging**

Intracellular Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_{i}\)) is a major signal for controlling a wide variety of cellular processes in addition to the cell cycle control. Ca\(^{2+}\) signaling is not only involved in the cell cycle control but also in the regulation of gene expression, cell differentiation, and apoptosis. In yeast, Ca\(^{2+}\) signaling is regulated by calcium/calmodulin-dependent protein kinases (CaMKs), which are activated by the elevation of intracellular Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_{i}\)) ([1]).

**Gene Disruption and Strain Construction**—The zds1Δ, cnb1Δ, sir2Δ, and sir3Δ strains were constructed by gene replacement. Genomic DNA was isolated from the zds1Δ::kanMX4, cnb1Δ::kanMX4, sir2Δ::kanMX4, and sir3Δ::kanMX4 strains on a BY4741 background (Invitrogen). The amplified fragment was used to transform a strain. The oligonucleotide primer sequences used in this study are available upon request.

**Yeast RLS—**RLS analyses were determined as described previously (30). Mother cells were discarded and buds were used as starting virgin cells. The RLSs of these cells were determined by noting and removing all subsequent daughters that were generated. All RLS analyses in this study were carried out on YPD plates with or without CaCl\(_2\) at least three times independently. Statistical significance was determined by performing a log rank test. Average RLSs were taken to be significantly different at \(p < 0.05\). Results from a single experiment are shown.

**β-Galactosidase Assays—**Cells carrying pKC201 (31) were grown to mid-log phase at 28 °C in defined minimal medium (synthetic complete (SC)) lacking uracil. The cells were harvested and resuspended in fresh YPD medium (pH 5.5). After incubation for 4 h at 28 °C with shaking, the cells were provided for assay of β-galactosidase activity as described previously (32). Error bars represent mean ± S.D. Significance levels for comparisons between the wild-type and other strains were determined with \(t\) tests.

**RESULTS**

**Deleting SIR3 Confers Ca\(^{2+}\) Resistance to zds1Δ Cells—**The growth of ZDS1 null mutant (zds1Δ) yeast cells in medium containing 300 mM CaCl\(_2\) is severely inhibited, with the cells exhibiting G1 arrest and highly polarized bud growth. As shown in supplemental Fig. 1, the growth inhibition and polarized bud growth but not the G1 delay of zds1Δ cells because of exogenous CaCl\(_2\) were suppressed by an additional szc14 mutation. The plasmid that complemented the phenotype of the szc14 mutants was cloned, and the mutant gene was identified. Genetic linkage analysis showed that the szc14 mutation resided in the SIR3 gene (data not shown). The deletion of the SIR3 gene, similar to the szc14 mutation, suppressed the growth inhibition and the polarized bud growth of zds1Δ cells, suggesting that the szc14 mutation is a loss of function allele of the SIR3 gene (Fig. 1, A–C). Therefore, we used the sir3 deletion (sir3Δ) in further experiments.

Why did the sir3Δ mutation suppress only the Ca\(^{2+}\)–induced hyperpolarized bud growth but not the G1–delay? We showed previously that the Ca\(^{2+}\) signal induces polarized bud growth by elevating the level of G1-cyclin Cln2 (35). To investigate whether the sir3 deletion affected the amount of Cln2, we examined the protein level of Cln2 by performing a Western blot analysis. For this purpose, we used cells containing the chromosomally integrated constructs for HA-tagged Cln2. The Cln2 levels in the presence and absence of external CaCl\(_2\) were decreased by the sir3Δ (compare zds1Δ and zds1Δ sir3Δ in Fig. 1D). The suppression of the Ca\(^{2+}\) sensitivity of the zds1Δ strain by the sir3 deletion was thus caused by a defect in the elevation of the Cln2 level in response to Ca\(^{2+}\). Basically, a similar effect of the sir3Δ disruption on the Cln2 level was seen on the WT background (compare WT and sir3Δ in Fig. 1D). These results indicate that the sir3Δ caused a defect in the elevation of the Cln2 level. The Sir3 protein in budding yeast is required for telomere silencing (15) and affects the life span (20, 24, 36). Therefore, in this study, we investigated a relationship between Ca\(^{2+}\)–signaling and life span.

**Increase in External and Cellular Ca\(^{2+}\) Levels Causes a Shortened RLS—**We first investigated whether the level of external Ca\(^{2+}\) would affect the RLS. Yeast cells undergo asymmetric divisions, producing a smaller daughter cell from a larger mother cell, and yeast aging is measured as a RLS, i.e. the number of times a mother cell can divide before it dies (37–39). Cells grown in the YPD medium supplemented with 100 mM CaCl\(_2\) had a shortened RLS relative to those grown on YPD (Fig. 2A). To check the specificity of Ca\(^{2+}\) on the RLS, we examined the effect of MgCl\(_2\) and KCl on the RLS because some cations (such
as Na\(^{+}\), Li\(^{+}\), Mn\(^{2+}\), Co\(^{2+}\), and Ni\(^{2+}\)) but not Mg\(^{2+}\) and K\(^{+}\) have been shown to affect the Ca\(^{2+}\) homeostasis (40, 41). As the result, neither MgCl\(_2\) (100 mM) nor KCl (150 mM) altered the RLS of WT cells significantly (Fig. 2A). These results established that external Ca\(^{2+}\) reduced RLS.

It is possible that external Ca\(^{2+}\) causes the shortened RLS by activating intracellular Ca\(^{2+}\) signaling. To investigate this possibility, we measured the mean RLS of \(zds1\Delta\) cells, in which Ca\(^{2+}\) signaling pathways might be activated by an increase in internal Ca\(^{2+}\) level (25). As expected, the mean RLS of \(zds1\Delta\) cells was shorter than that of WT cells under the experimental conditions (Fig. 2B), although it was reported previously that the mean RLS of \(zds1\Delta\) cells was longer than that of WT cells (24).

To investigate the activation of Ca\(^{2+}\) signaling pathways in \(zds1\Delta\) cells under the experimental conditions, we estimated the calcineurin activity in WT and \(zds1\Delta\) cells under normal growth conditions (YPD) by \(\beta\)-galactosidase assay. The \(\beta\)-galactosidase activity in WT cells indicated the basal level of this reporter system because the activity in WT cells was comparable with that in the calcineurin-deleted cells (\(cnb1\Delta\)) (Fig. 2C). As expected, the \(\beta\)-galactosidase activity in the \(zds1\Delta\) cells increased significantly by 3-fold compared with that in WT cells (Fig. 2C), and this increase was indeed dependent on calcineurin (Fig. 2C, compare \(zds1\Delta\) and \(zds1\Delta\ cnb1\Delta\)). These results indicate that the increase in cellular Ca\(^{2+}\) level in the \(zds1\Delta\) cells causes the shortened RLS by activating Ca\(^{2+}\) signaling pathway(s).

Increase in Cellular Ca\(^{2+}\) Level by PMR1 Deletion Shortened RLS—We previously isolated two genes, PMR1 (42, 43) and PMC1 (44, 45), encoding Golgi and vacuolar Ca\(^{2+}\)-ATPases, respectively, as multicopy suppressors of Ca\(^{2+}\) sensitivity of the \(zds1\Delta\) cells (46). Both Pmr1 and Pmc1 transport Ca\(^{2+}\) from the cytosol into internal compartments. In addition, it has been reported that a lack of Pmr1 results in alterations in cellular Ca\(^{2+}\) homeostasis, including an increased rate of cellular Ca\(^{2+}\) uptake from the extracellular environment and an enhanced sensitivity to high extracellular Ca\(^{2+}\) levels (47, 48).

To confirm that an increase in cellular Ca\(^{2+}\) shortens the RLS in yeast, we examined RLS of the \(pmr1\)- and \(pmc1\)-deleted cells.
The pmr1Δ cells but not the pmc1Δ showed a severe reduction in mean RLS of ~64% (Fig. 2D), indicating that Pmr1-mediated Golgi Ca²⁺ sequestration but not a Pmc1-mediated vacuolar one is important for longevity under this experimental condition. To further to investigate the contribution of these Ca²⁺-ATPases in cellular Ca²⁺ homeostasis, we measured the calcein activity in these cells with the reporter assay. Consistent with the result of RLS, β-galactosidase activity in the pmr1Δ cells but not in the pmc1Δ significantly increased by 4-fold compared with the basal level in the WT cells (Fig. 2C). These results indicate that Pmr1-mediated Ca²⁺ sequestration to the Golgi plays an important role in regulation of RLS through cellular Ca²⁺ homeostasis.

Hyperactivation of Calcineurin Reduces the RLS—To further confirm the above suggestion that activation of Ca²⁺ signaling pathways shortens the RLS, we investigated whether overexpression of the constitutively activated calcineurin (Cmp2ΔC, C-terminal autoinhibitory domain-truncated catalytic subunit) promotes aging and shortens the RLS. This was indeed the case. The WT cells expressing the activated calcineurin (YEp24-Cmp2ΔC) showed a reduced RLS compared with the WT cells carrying the empty plasmid (YEp24) (Fig. 3A). This result established that activation of calcineurin signaling shortens the RLS.

Calcineurin Deletion Overcomes the Shortened RLS of zds1Δ Cells—We reported previously that calcineurin and Zds1 play an antagonistic role in the regulation of cell growth and morphogenesis in the presence of high Ca²⁺ (25). As shown in Fig. 2B, deletion of ZDS1 resulted in about 20% decrease in the mean RLS with hyperactivation of calcineurin. We investigated whether calcineurin deletion overcomes the shortened RLS in zds1Δ cells. As expected, the mean RLS of zds1Δ cnb1Δ double deletion cells was longer than that of zds1Δ single deletion cells (Fig. 3B). Interestingly, the mean RLS of zds1Δ cnb1Δ cells was comparable with that of the WT cells. These results indicate that calcineurin and Zds1 play an antagonistic role in the regulation of RLS and that the activated calcineurin promotes aging in the zds1Δ cells.

Calcineurin and Sir3 Act Redundantly in the Regulation of RLS—We next examined the effect of the calcineurin deletion on the RLS of WT cells. Surprisingly, the calcineurin deletion per se on the WT background reduced the RLS under normal growth conditions (Fig. 3, B and C), suggesting that the basal
activity of calcineurin is required for the maintenance of the RLS under this condition.

In the Sir3-mediated regulation of RLS, the Mpk1 MAPK cascade was identified as the pathway that phosphorylates Sir3, which leads to the shortening of the RLS (49). We and others showed earlier that calcineurin and Mpk1 act redundantly in cellular events (25, 50, 51). To investigate whether calcineurin is related to the Sir3-mediated regulation of RLS, we examined the RLS of the double deletion mutant (Fig. 4A). No change was observed in the growth of mutant cells grown on -URA medium plates as in WT cells (Fig. 4A). No change was observed in the growth of cnnb1Δ cells on -URA medium plates as in WT cells (Fig. 4A), indicating that calcineurin did not affect the silencing at a DNA region.

Next, to investigate whether calcineurin was required for silencing at other heterochromatin regions, we examined silencing at telomeres and the mating-type locus in the cnnb1Δ-deleted cells. We constructed the CNB1-deleted cells, in which the reporter gene was strongly silenced at either the NTS1 or NTS2 sites in WT cells, as indicated by poor growth on SC minus uracil (-Ura) medium compared with the same reporter negative control. Cells were spotted onto complete medium as a plating and growth control or onto medium supplemented with 5-fluoroorotic acid, a drug that is toxic to cells that are deficient in uracil biosynthesis. The cells were plated on 5-fluoroorotic acid medium and grew on the 5-fluoroorotic acid medium, whereas the empty plasmid (YEp24) or YEp24-CMP2ΔC plasmid grown on YPD at 25 °C were examined. Mean RLSs are YEp24, 25.5 (n = 40) and YEp24-CMP2ΔC, 21.9 (n = 39); RLS analysis of WT, zds1Δ, cnnb1Δ, and zds1Δ cnnb1Δ mutant cells grown on YPD at 25 °C. The mean RLSs are WT, 26.9 (n = 44); zds1Δ, 21.8 (n = 44); cnnb1Δ, 23.5 (n = 44); and zds1Δ cnnb1Δ, 27.6 (n = 43). C, RLS analysis of WT, cnnb1Δ, sir3Δ, and cnnb1Δ sir3Δ mutant cells grown on YPD at 25 °C. The mean RLSs are WT, 25.2 (n = 18); cnnb1Δ, 19.7 (n = 33); sir3Δ, 19.6 (n = 20); and cnnb1Δ sir3Δ, 15.0 (n = 36).
(rDNA and telomeres), the silencing at the mating-type locus was normal in the Δcnb1 cells (Fig. 4C). These results indicate that calcineurin is not important for these genes silencing and plays an important role in a novel regulation of the RLS.

**DISCUSSION**

In conclusion, we reported that calcineurin plays an important role in regulating the RLS/aging in budding yeast. Hyper-activation of calcineurin signaling reduced the RLS. Indeed, the shortened RLS of zds1Δ cells, in which calcineurin is activated by the increase in cellular Ca\(^{2+}\) level, was suppressed by the calcineurin deletion. These results indicate that the constitutive activation of calcineurin would be harmful to yeast in the long term, although it could enhance resistance to several stresses, including ionic stress (Na\(^+\), Li\(^+\), Mn\(^{2+}\)), high pH, and the presence of cell wall-disrupting compounds (calcofluor-white and Congo red). Surprisingly, loss of function of the calcineurin gene also promoted the aging in the WT cells, indicating that basal activity of calcineurin is required for maintenance of RLS under normal growth conditions. However, in the multicellular organism *Caenorhabditis elegans*, a calcineurin-deficient mutant displayed an extended lifespan (5, 6). It is possible that an optimal level of calcineurin activity/expression would be important for longevity in the unicellular organism yeast.

How do calcineurin and Zds1 act in the regulation of the RLS? Our results suggested that calcineurin and Zds1 play an antagonistic role in the regulation of RLS. We reported previously that the polarized bud growth, the defect of cell proliferation, and the G\(_2\) delay caused by high calcium in the zds1Δ strain were abolished by the calcineurin deletion mutations (25). In addition, calcineurin activity was enhanced in the zds1Δ cells (Fig. 2D). Therefore, it is possible that Zds1 may be required for the longevity in the negative regulation of calcineurin. This possibility needs to be studied further.

In this study, we showed that the zds1Δ deletion reduced the RLS. However, previously, the another group reported that the zds1Δ cells led to an extended RLS (24). Although both studies used the W303 strain, the mean RLS of their WT cells (~20 times) was much shorter than that of our WT cells (25~27 times), indicating that both experimental conditions are different from each other. The contradiction of RLS in the zds1Δ cells would be caused by the differences in the experimental conditions. Further, it was reported that the zds1Δ strain on the BY4742 background had no effect on the RLS (54). We noted that the zds1Δ cells on the W303 background were more sensitive to Ca\(^{2+}\) than those on the BY4742 background. It is possible that the short-lived zds1Δ cells on W303 were due to the elevated level of intracellular Ca\(^{2+}\).

Why did the pmr1Δ cells exhibit a severe reduction in mean RLS compared with other cells (zds1Δ, cnb1Δ, and sir3Δ)? The

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3 R. Tsubakiyama, M. Mizunuma, and D. Hirata, unpublished results.
Pmr1 protein plays an important role in both secretion and proton transport (55, 56). Although the gene that is related to the secretory pathway has not been identified yet in the systematic genetic screening for the RLS regulator, it has been reported that some proton ATPases mutants, such as the VMA genes encoding vacuolar H^+_ATPases, reduced the RLS (57). These results suggest that the severe RLS reduction of pmr1A cells was derived not only from alternation of the Ca^{2+} homeostasis but also from defect of proton transport. As the double deletion between calcineurin and the VMA gene shows synthetic lethality (51, 58), the shortened RLS of the calcineurin deletion might be caused at least in part by the impairment in control of the intracellular pH. Although a functional interaction between secretion and RLS has not been shown, we have not ruled out this possibility.

We showed previously that calcineurin leads to the Cln2 upregulation by a mechanism that is mediated by the degradation of Yap1 (46). We showed that Sir3 was required for the elevation of the Cln2 level in the regulation of the RLS, a functional link between Sir3 and Cln2 has not yet been identified. However, our results suggest the possibility that the optimum levels of Cln2 might be important for longevity. Our findings also suggest that Ca^{2+} homeostasis is necessary for longevity in yeast.

It will be interesting to determine whether altered Ca^{2+} homeostasis influences the life span of higher eukaryotes and whether the corresponding pathways are a part of a conserved network involved in the control of biological aging.

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Supplementary Fig. 1. The scz14 mutation, a mutant allele of SIR3 gene, suppresses various phenotypes of the zds1Δ strain. (A) Effect of scz14, mutant alleles of SIR3, on the growth of the zds1Δ mutant strain on solid medium. WT, scz14, and zds1Δ scz14 cells were spotted on YPD solid medium containing 300 mM CaCl₂, after which the plates were incubated at 25°C for 2 days. (B, C) Cell morphology (DIC: Differential Interference Contrast) after 6 h of incubation with 100 mM CaCl₂ at 25°C (B). Flow cytometry analysis (FACS) of PI-stained cells of various strains after 6 h of incubation with 100 mM CaCl₂ at 25°C (1C: 1 DNA copy or 2C: 2 DNA copies) (C).
Evidence for antagonistic regulation of restart from G₁ delay in response to osmotic stress by the Hog1 and Whi3 in budding yeast.
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Evidence of Antagonistic Regulation of Restart from G₁ Delay in Response to Osmotic Stress by the Hog1 and Whi3 in Budding Yeast

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Hog1 of Saccharomyces cerevisiae is activated by hyperosmotic stress, and this leads to cell-cycle delay in G₁, but the mechanism by which cells restart from G₁ delay remains elusive. We found that Whi3, a negative regulator of G₁ cyclin, counteracted Hog1 in the restart from G₁ delay caused by osmotic stress. We have found that phosphorylation of Ser-568 in Whi3 by RAS/cAMP-dependent protein kinase (PKA) plays an inhibitory role in Whi3 function. In this study we found that the phosphomimetic Whi3 S568D mutant, like the Δwhi3 strain, slightly suppressed G₁ delay of ∆hog1 cells under osmotic stress conditions, whereas the non-phosphorylatable S568A mutation of Whi3 caused prolonged G₁ arrest of ∆hog1 cells. These results indicate that Hog1 activity is required for restart from G₁ arrest under osmotic stress conditions, whereas Whi3 acts as a negative regulator for this restart mechanism.

Key words: G₁ cyclin; Hog1; osmotic stress; RAS/cAMP-dependent protein kinase; Whi3

Extracellular stresses activate stress-activated protein kinases (SAPKs), which regulate transcription of the genes relevant to adaptation to various forms of stress. The resulting activated SAPKs transiently arrest the progression of the cell cycle until the cells have adapted to their new environment. This adaptation mechanism is important for resumption of the cell cycle. In budding yeast, the Hog1 SAPK pathway mediates adaptation to high osmolarity. The mechanism activating the Hog1 pathway is well understood.¹ Hog1 is also required for cell-cycle regulation after exposure to hyperosmotic stress.²⁻⁴ For instance, activated Hog1 induces G₁ cell-cycle arrest by a dual mechanism that involves downregulation of G₁ cyclin transcription and direct phosphorylation of CDK-inhibitor Sic1,⁵ but the mechanism by which the cells restart from G₁ delay remains elusive.

Whi3 was originally identified as a positive regulator of cell-size control and was found to be involved in the regulation of Start.⁶ The WHI3 gene encodes an RNA-binding protein that negatively regulates CLN3 preventing the G₁/S phase transition, and Whi3 functioning is required for developmental options such as invasive growth and meiosis.⁶⁻⁹ Recently we reported that phosphorylation of Ser-568 in Whi3 by RAS/cAMP-dependent protein kinase (PKA) plays an inhibitory role in Whi3 functioning.¹⁰ These findings suggest that this process of phosphorylation plays a critical role in commitment to the cell division cycle and to the alternative developmental outcomes, such as meiosis and invasive growth.

The PKA pathway in yeast has a role in coordinating responses to nutrient status and various forms of stress.¹¹,¹² In response to certain environmental cues, enzyme Cyr1/Cdc35 adenyllyl cyclase produces cAMP, which then binds to the PKA regulatory subunit (Bcy1), which represses activation of the catalytic subunits of PKA (Tpk1/Tpk2/Tpk3) under normal conditions.¹³,¹⁴ cAMP-bound Bcy1 is released from the Tpk proteins, which then activate or repress downstream transcription factors and thus respond to signaling, but although this PKA signaling pathway has been well characterized, its involvement in the osmotic-stress response is not fully understood.

In this study, we identified Whi3 as a suppressor mutant of the osmotic sensitivity of hog1 mutants. Our results indicate that Hog1 and Whi3 antagonize the regulation of restart from G₁ delay in response to osmotic stress.

Materials and Methods

Yeast strains and media. The Saccharomyces cerevisiae strains used in this study are listed in Table 1. The media used were as described previously.¹⁵

Gene disruption and strain construction. Deletion of the genomic CLN1, CLN2, and CLN3 genes (CLN1, 2, and 3 plasmids provided by I. Yamashita) were performed by means of a disruption plasmid.

Quantitative reverse transcription real-time PCR (RT-qPCR). RT-qPCR analysis of total RNA was done in a LightCycler (Roche Applied Science, Tokyo, Japan) with a One Step SYBR PrimeScript

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Abbreviations: PKA, RAS/cAMP-dependent protein kinase; RT-qPCR, quantitative reverse transcription; SAPK, stress-activated protein kinase; SD, synthetic dextrose; WT, wild type; YPD, yeast peptone dextrose
RT-PCR Kit II (Takara, Kyoto, Japan). Total RNA was isolated by use of an RNaseasy MiniKit (Qiagen, Tokyo, Japan) following the manufacturer's instructions. The relative amount of the TPK1 mRNA was normalized to that of ACT1 mRNA in three independent experiments. Error bars were used to show mean ± SEM. The value for WT at time 0 is referred to as “1.” The oligonucleotide primer sequences used in this study are available on request.

Results

Isolation of the sgh4/whi3 mutation as a suppressor of the osmotic sensitivity of Δhog1 strain

Deletion of the HOG1 gene causes severe growth retardation in yeast on solid media containing 150 mM CaCl2 due to hyper-activation of Ca2+-signaling and high osmosality.16,17 To identify the genes involved in this regulatory mechanism, we screened for mutants that suppress the growth defect of a hog1-deleted (Δhog1) strain on solid medium containing a high concentration of CaCl2. Recessive, Ca2+-resistant mutants were classified into six loci, designated sgh1-sgh6 (for suppressors of Ca2+-induced growth defect of hog1). Although these mutations were originally isolated based on their ability to suppress the CaCl2 sensitivity of Δhog1 cells, four mutations (sgh3/sup1, sgh5/skd1, sgh6/cyc8, and sgh4), but not two others (sgh1/swel and sgh2/cnb1), have been found to suppress also the osmotic sensitivity (0.6 M sorbitol and 0.4 M KCl) of the hog1 mutant.7,18 In this present study, we characterized mutation sgh4.

By crossing sgh4 Δhog1 strain with the parental strain, the sgh4 strain was constructed. The cell sizes of the sgh4 strain and of the sgh4 Δhog1 strain was smaller than that of wild-type (WT), and exhibited temperature sensitivity at 37°C (data not shown). Cloning and sequencing of the gene that complemented the temperature sensitivity of the sgh4 strain suggested that this mutation is an allele of the WHI3 gene (data not shown). Integrating linkage analysis indicated that the sgh4 mutation was located within or close to the WHI3 gene.6 Deletion of the WHI3 gene, similarly to the sgh4 mutation, suppressed the osmotic sensitivity of Δhog1-strain cells (Fig. 1A, lanes 2 and 5; reference 17), suggesting that the sgh4 mutation is a loss-of-function mutant allele of the WHI3 gene. In further experiments, we studied the whi3-deletion (Δwhi3) mutation.

Inhibition of Whi3 suppressed the osmotic sensitivity of hog1 by upregulating Cln3

The best characterized function of Whi3 is its ability to inhibit the Cln3 function in the G1 phase by binding to CLN3 mRNA, thereby inhibiting the Cln3-Cdc28 mediated activation of two transcription factors, SBF (Swi4-Swi6) and MBF (Mbp1 and Swi4), which drive the expression of G1 cyclins CLN1 and CLN2, which leads to a delay in the start of the cell cycle.2,8,10,18 Whi3 has been to bind a large number of mRNAs functionally related to the endoplasmic reticulum,9 but the involvement of Whi3 in the osmotic stress response is unclear. First we examined, whether the suppression of the osmotic sensitivity of Δhog1 cells by the whi3 deletion was caused by upregulation of Cln3. As expected, the suppression of the osmotic sensitivity of Δhog1 strain by the whi3 deletion was cancelled by additional deletion of the CLN3 gene (Fig. 1A, lane 8). A similar effect was also observed for the deletion of other G1 cyclin genes, CLN1 and CLN2 (Fig. 1B, lane 5). Consistently with the results of whi3 deletion, overexpression of the CLN2 gene but not that of the CLB cyclin genes suppressed the osmotic sensitivity of the Δhog1 strain (Fig. 1C). Moreover, the Δcln3 and Δwhi3 Δcln3 strains did not show osmotic sensitivity (Fig. 1A, lanes 4 and 7), confirming that the cln3 deletion itself did not affect osmotic sensitivity. Together, these results suggest that the Hog1 pathway positively regulated G1 cyclin, whereas Whi3 negatively regulated G1 cyclin, and that this mechanism is important for cell growth under osmotic conditions (Fig. 1E).

Since the WHI3 gene was originally identified as a positive regulator of cell-size control,6 we further investigated whether inhibition of the Cln3 function by Whi3 participates in cell-size control under osmotic conditions. Exponentially growing WT and Δhog1 cells in normal YPD medium were similar in size, but under osmotic conditions (a medium containing 0.4 M KCl), the Δhog1 cells were larger than the WT cells (Fig. 1D). This increase in size of the Δhog1 cells was reversed by the whi3 deletion (Fig. 1D, Δhog1 Δwhi3), further, an additional cln3 deletion in the Δhog1 Δwhi3 double mutant again caused an increase in cell size (Fig. 1D). These results indicate that the increase in cell size of Δhog1 under osmotic conditions was caused by Whi3-

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Table 1. Strains Used in This Study

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dependent downregulation of G₁ cyclin. Thus the regulation of G₁ cyclin via Whi3 is also important for cell-size control under such stress conditions.

**Inhibition of Whi3 was required for restart from osmotic stress-induced G₁ delay**

To clarify the role of the regulatory pathway of G₁ cyclin via Whi3 in cell-cycle control under osmotic conditions, we examined carefully the cell-cycle progression of cells released from the synchronized G₁ phase with α-factor in YPD medium containing either of two concentrations of KCl (0.4 and 0.6 M). Under osmotic conditions (0.4 M KCl), both WT and Δhog1 cells exhibited delays in G₁/S transition (Fig. 2A and B), bud formation, and the onset of nuclear division (Fig. 2C). As previously reported, osmotic stress-induced G₁ delay in the Δhog1 cells was longer than that in the WT cells, suggesting that Hog1 is required for restart from G₁ delay (Fig. 2B). Further, deletion of the WHI3 gene partially suppressed G₁ delay in the Δhog1 strain (Fig. 2B). This suggests that when Whi3 is inhibited, cells can restart from osmotic stress-induced G₁ delay. Moreover, when we used a higher concentration of KCl (0.6 M), the G₁/S transition of Δwhi3 cells was slightly accelerated as compared to that of the WT cells (Fig. 2D, 90–105 min). This indicates that Whi3 plays an important role in G₁ cell-cycle control under osmotic stress conditions.

**The phosphomimetic Whi3-S568D mutation slightly suppressed the defect in restart from the osmotic stress-induced G₁ delay of the hog1 mutant**

Recently, we reported that Ser-568 in Whi3 is phosphorylated by PKA and that this plays an inhibitory role in Whi3 functioning. It has also been reported that PKA is upregulated at the transcriptional level in response to environmental stresses such as heat shock. To determine whether PKA is upregulated by osmotic stress, we examined the transcription level of the PKA gene TPK1 in WT cells after exposure to osmotic stress (0.4 M KCl) by quantitative RT-PCR (RT-qPCR). The level of TPK1 mRNA was conspicuously elevated within 30 min of exposure to osmotic stress, and then dropped to the basal level after 60 min (Fig. 3). Hog1 is
Fig. 2. Inhibition of Whi3 Was Required for Restart after Osmotic Stress-Induced G1 Delay.

A and B, Cellular DNA levels of the WT, ∆whi3, ∆hog1, and ∆hog1 ∆whi3 strains synchronized with α-factor and released into YPD medium with and without 0.4 M KCl at 28°C. C, The kinetics of the progression of these cell-cycle events is shown. WT, ∆hog1, ∆whi3, and ∆hog1 ∆whi3 strains synchronized with α-factor were released into YPD medium with and without KCl. Quantification of cumulative percentages of bud formation and nuclear division during cell-cycle progression in the strains synchronized with α-factor were determined periodically by microscopic observation and propidium iodide staining respectively. At least 300 cells were counted for each time point. D, Cellular DNA levels of the WT and ∆whi3 strains synchronized with α-factor and released into YPD medium with 0.6 M KCl. Solid triangles indicate the period of G1/S transition for each strain.
well established as a transcriptional regulator in the osmotic stress response. To investigate further whether the transcriptional activation of TPK1 in response to osmotic stress was dependent on Hog1, we performed the same experiment using Δhog1 cells. The results indicated that the TPK1 mRNA level in the Δhog1 cells rose more slowly, and that the TPK1 mRNA level was significantly lower at each time point than that in the WT cells (Fig. 3). This indicates that PKA was quickly activated by the osmotic signal in a Hog1-dependent manner.

Because previous work has shown that phospho-mimic Whi3-S568D mutant cells behave like Δwhi3 cells, we speculated that the Whi3-S568D mutation would suppress the G1 delay of Δhog1 cells in response to osmotic stress. To test this possibility, we examined the effects of phospho-mimicking S568D and non-phosphorylatable S568A mutations of Whi3 on the cell-cycle progression of Δhog1 cells under osmotic stress conditions (0.2 and 0.6 M KCl). As reported previously, under normal growth conditions, the Whi3-S568D single mutation led to a shortened G1 phase as in the case of Δwhi3 cells, whereas the Whi3-S568A single mutation led to a prolonged G1 phase (data not shown). As expected, the Whi3-S568D mutation slightly suppressed the G1 delay of Δhog1 cells in a medium containing 0.2 M KCl (Fig. 4A, arrowheads, compare to Δhog1 Whi3 and Δhog1 Whi3-S568D for 45–60 min). We noted that the suppressive effect of the Whi3-S568D mutation on the

![Fig. 3. TPK1 mRNA Was Transiently Upregulated by Osmotic Stress in a Hog1-Dependent Manner.](image)

Fig. 3. TPK1 mRNA was transiently upregulated by osmotic stress in a Hog1-dependent manner. TPK1 and ACT1 (control) mRNA levels in WT and Δhog1 cells were determined by RT-qPCR. Cells were grown at 28°C in YPD medium containing 0.4 M KCl. The relative intensity of the TPK1 mRNA was normalized to that of ACT1 mRNA. The value for the WT at time 0 was set at 1.

![Fig. 4. The Phosphomimetic Whi3-S568D Mutation Slightly Suppressed the G1 Delay in hog1 Mutant upon Exposure to High Osmolarity.](image)

Fig. 4. The phosphomimetic Whi3-S568D mutation slightly suppressed the G1 delay of Δhog1 cells in a medium containing 0.2 M KCl (Fig. 4A, arrowheads, compare to Δhog1 Whi3 and Δhog1 Whi3-S568D for 45–60 min). We noted that the suppressive effect of the Whi3-S568D mutation on the
cell-cycle progression of the Δhog1 cells was much weaker than that of whi3 deletion (compare Figs. 2B and 4A). Further, the Whi3-S568A mutation caused prolonged G1 arrest of Δhog1 cells in a medium containing 0.6 M KCl (Fig. 4B), as for Whi3 wild-type cells under osmotic stress conditions (Fig. 2B, Δhog1 cells). Together, these observations suggest that inhibition of Whi3 is required for restart from osmotic stress-induced G1 delay.

Discussion

We suggest that Hog1 SAPK pathway and the Whi3 cell-cycle regulator antagonize restart from G1 delay following osmotic stress. Upon exposure of Δhog1 cells to osmotic stress, the cells exhibited a severe G1 delay, which was suppressed by the whi3 deletion and by overexpression of the CLN2 gene (Figs. 1A–C and 2A–C). These results suggest that the G1 delay was caused by a low level of G1 cyclins through activation of Whi3, a negative regulator of G1 cyclin. Thus our results suggest that Hog1 activity is important for restart/recovery from G1 arrest under high-osmolarity conditions. On the other hand, Whi3 acts as a negative regulator for this restart mechanism. Thus, Hog1 and Whi3 antagonize the regulation of restart from G1 delay caused by osmotic stress (Fig. 1E). Transcription of TPK, a budding yeast PKA Tpk1 that directly phosphorylates and inactivates Whi3,16 was upregulated under osmotic stress in a Hog1-dependent manner (Fig. 3). Further, the phospho-mimicking Whi3 S568D mutant slightly suppressed the G1 delay of Δhog1 cells under high-osmolarity conditions (Fig. 4A). Thus, it is possible that Hog1 is involved in the restart from osmotic stress-induced G1 delay in that it downregulates Whi3 through transcriptional activation of PKA/TPK1. Alternatively, it is possible that Whi3 plays a role in restart independently of Hog1 or Tpk1. Thus our results raise important questions: Is Whi3 indeed phosphorylated by PKA upon osmotic stress, and is it dependent on Hog1? These possibilities must be clarified further.

Our data are consistent with previous results indicating the involvement of Hog1 in recovery from transient G1 arrest in response to moderately hyperosmotic stress.30 It has been reported that Hog1 activation is required for G1 arrest in response to hyperosmotic stress.35 Here, we found that a G1 delay was induced by osmotic stress even in Δhog1 cells (Fig. 2B, Δhog1), indicating that the cells have a Hog1-independent mechanism inducing G1 delay in response to osmotic stress. Hog1 regulates a number of molecules in cell-cycle control (both G1 arrest and restart from G1) under osmotic stress conditions. A previous study indicated that expression of a constitutively active form of Hog1 is capable of inducing G1 arrest under normal growth conditions,36 suggesting that a Hog1-induced mechanism for G1 arrest might be dominant to that for restart from G1.

The partial rescue of the sensitivity of Δhog1 strains to osmotic stress by deletion of the WHI3 gene (Fig. 1A) indicates that Hog1 regulates other molecules, in addition to Whi3, for growth inhibition under these conditions. It is possible that the G1 delay in Δhog1 cells under osmotic stress is related to a general inhibition of the initiation of translation by osmotic stress.20

It was found recently that Hog1 is involved in the regulation of exit from the resting state,21 but the target of Hog1 in this system is obscure. It would be interesting to determine whether Hog1-dependent or Hog1-independent Whi3 regulation operates in this system.

Acknowledgments

We thank Dr. Ichiro Yamashita for materials. We feel deeply appreciative towards Professor Emeritus Tokichi Miyakawa of Hiroshima University for insightful discussion and his comments on the manuscript. This work was supported in part by the program Grants-in-Aid for Scientific Research from the Japan Society for the Promotion of Science (to M.M. and D.H.). R.T. is the recipient of a JSPS Fellowship (DC2).

References

バイオミディア

パン酵母でアンチエイジング

椿山 諒平*・水沼 正樹

老化は誰にでも起る現象であり、どんなに補い人でも避けることはできない。人類は昔から不老不死を夢見てきたが、果たしてそんなことは可能なのかだろうか？
その夢を実現するキーワードはまだ発見されていない。少なくなても老化を遅らせることはできるよう。というのも、複雑な老化的メカニズムが徐々に明らかになってきたからである。

人の寿命がどのように決まるのか明らかにするうえで、酵母、線虫、マウスといったわざわざ真核モデル生物を用いた研究が必要不可欠である。実際に、さまざまなモデル生物を用いた研究において、その謎のひとつ一つが解き明かされてきた。特に、出芽酵母を用いた研究により得られた知見は多い。老化を理解するための最もシンプルなモデル生物のひとつとして用いられている。
出芽酵母は、パン酵母、ビール酵母といわれる微生物であり、私たちはにも菌類らしい生物である。しかしながら、そもそも出芽酵母が老化するのはという疑問をもつつ人も多いかもしれない。出芽酵母には、「経時的生命」、「複製的生命」、「死の生命」など2つの生命があり、1つの細胞が一生の間に分裂できる回数に限界があり、最終的には加齢により細胞を産むことができなくなる（複製的生命）。また、分裂しない細胞でも、その生存率は日を追うことに減少して行く（経時的生命）。これらが出芽酵母の老化であり、老化に伴って生じる細胞機能が衰えていく。つまり、出芽酵母も人と同じように老いていて、最終的には死を迎える。

出芽酵母の老化は、細胞によって有害な物質の蓄積によって引き起こされるといわれている。環境のリポソームDNA（ERC）や、酸化または凝集し難かったタンパク質などがその例であり、老化にともなってこれらの物質が細胞内に蓄積することが知られている。出芽酵母のリポソーム遺伝子は100以上の繊細な配列で構成されたeDNA領域と呼ばれる部分に存在する。これに起因した「コニシモームによって生じる物質がERCという物質である。実際、ERCの形成を防ぐ及びの状況をもたらすと生命を延ばすことができ、一方その抑圧を促すFob1を欠損させることによって寿命の延長が報告されている）。出芽酵母は、その名の通り出芽によって増殖し、大きい母細胞、小さい娘細胞といったように非対称的に分裂する。驚くことに、実は細胞の中の非対称に分裂することが明らかとなっている。細胞が分裂するとき、母細胞に蓄積した老化の原因となるERCなどの物質が娘細胞には受け渡されないような仕組みが存在するのである。これにより、娘細胞は完全に若返ることができる。つまり、生まれてきた娘細胞は老化をリセットできるのだ。この機構は、なにも出芽酵母に限ったことではない。幹細胞や生殖細胞など子孫を残すために必要な細胞でも、老化のリセッティングが観察されており、ヒトにおいても同様の機構が存在することが考えられている。

老化を遅延させることで生命を延ばす方法で、酵母をはじめとするさまざまなモデル生物や、最近ではサルにおいてもその効果が確認されている。出芽酵母では、培地に含まれるグルコースを2%から0.5%に減らすと約30%の寿命延長効果が得られる。カリカリを摂取することで寿命が延長する仕組みについてはまだ不明な点も多いが、少なくとも出芽酵母においては、TORキナーゼやプロテインキナーゼA、Sch9キナーゼなどを介した栄養応答シグナル伝達系が関与していることが明らかとなっている。栄養応答シグナルとは、生育に必要な細胞内の栄養状態を細胞内に伝える手段であり、その状態に最も適した細胞応答を誘導する。たとえば、このシグナルの減少は、ストレスへの耐性、翻訳レベルの低減、オートファジー（細胞自身の構成成分を分解する現象）の誘導など細胞にとって有利な応答につながる。さらに、寿命の延長をもたらすことも知られている。実際、出芽酵母の流れがシグナル伝達経路が欠損し変異株は寿命が延長することができる知見が知られているが、これらの変異株にカリカリ制限を行っても、寿命がさらに延長することはない。また、カリカリ制限の際に、これらのシグナル伝達系の活性が減少することも知られている。これらの報告は、栄養応答シグナルを減少させることでカリカリ制限の効果と同じ効果をもっていることを裏付ける。

本稿では、出芽酵母を用いた研究により明らかとなった老化・寿命決定のメカニズムの一部を紹介した。
その知見のいくつかは出芽酵母に特異的なものである。しかしながら、栄養応答シグナル経路をはじめとした、寿命を制御する経路は驚くほど保存されており、高等生物においても存在することが確認されている。寿命を延ばすことができる生理活性物質も出芽酵母を用いて発見されており、これらの物質によるヒトの寿命延長の可能性について注目が集まっている。いつまでもパンを膨らませる道具にすぎないパン酵母であるが、そのパン酵母のおかげで健康的、長生きできる日も近いだろう。


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