博士論文

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．公表論文

(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.
Ryohei Tsubakiyama, Masaki Mizunuma, Anri Gengyo, Josuke Yamamoto, Kazunori Kume, Tokichi Miyakawa and Dai Hirata
The Journal of Biological Chemistry, 286 (33), 28681-28687 (2011)

３．参考論文

(1) Evidence for antagonistic regulation of restart from G_1 delay in response to osmotic stress by the Hog1 and Whi3 in budding yeast.
Masaki Mizunuma, Takafumi Ogawa, Tetsuya Koyama, Atsunori Shitamukai, **Ryohei Tsubakiyama**, Tadamasa Komaruyama, Toshinaga Yamaguchi, Kazunori Kume and Dai Hirata
Bioscience, Biotechnology, and Biochemistry, 77 (10), 2002-2007 (2013)

(2) パン酵母でアンチエイジング
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主論文

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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^{2+}-signaling pathways, Mpk1 MAP kinase and CN, control G_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^{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^{2+}-signaling pathways is involved in the regulation of life span, the relationship between Ca^{2+} and aging in yeast is not known.

Because both cAMP and Ca^{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^{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 *WHI3* was identified as a suppressor mutation of the Ca^{2+}-sensitive phenotypes of Δ*hog1* 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^{2+}-signaling and life span. The strain lacking Zds1, a negative regulator of Swe1, shows G_2 cell cycle arrest, and polarized bud growth, due to the activation of the cellular Ca^{2+}-signaling pathways. Here I show that this Ca^{2+}-signaling activated cells had decreased life span as well as the shortened life span by increased external Ca^{2+}. On the other hand, the deletion of *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 G_1 phase of the cell cycle. In the budding yeast *Saccharomyces cerevisiae*, this control network is called Start. The Start/G_1 phase is initiated by the G_1 cyclin Cln3, which associate with the cyclin-dependent kinase Cdc28. Previously, we identified a mutant allele of *whi3*, a negative regulator of the G_1 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 G_1 phase by binding to *CLN3* mRNA, thereby inhibiting the expression of the G_1 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 G_1/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 G_1 cell cycle arrest by a dual mechanism that involves down-regulation of the G_1 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 Ca^{2+} and the Hog1 pathways (Shitamukai *et al*., 2004). However, the relation between the Ca^{2+} signaling pathways and the Hog1 pathways still largely remain to be investigated.

The growth of Δ*hog1* cells is compromised on solid medium containing a high concentration of Ca^{2+}, exhibiting hyperpolarized bud growth and G_2 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 Ca^{2+} condition. The isolated mutants were classified into 6 loci designated as *sgh1-sgh6* (for suppressors of Ca^{2+}-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 G_1 cyclin *CLN1*, *CLN2* level by binding to *CLN3* mRNA. Thereby, Whi3 inhibits the association between G_1 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 G_1 cyclin *CLN1*, *CLN2* mRNAs. The G_1/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 (Garí *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 response 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 G_1 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 G_1/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 *Bam*HI and *Sal*I, and then cloned into the *Bam*HI- and *Sal*I- 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 *Bam*HI-*Sal*I fragment of the pMBP-WHI3-S568A plasmid was cloned into *Bam*HI- and -*Sal*I-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 *Apa*I-*Sph*I fragment of the pT-Whi3-5T-3HA plasmid was cloned into the *Apa*I- and *Sph*I- 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 Δ*whi3* strain on the W303 background was constructed by gene replacement. Genomic DNA was isolated from the Δ*whi3::kanMX4* strain on a BY4741 background (Invitrogen). The PCR-amplified fragments of Δ*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 *Nsp*V (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 $MnCl_2$ with or without phosphatase inhibitors (50 mM NaF and 5 mM Na_3VO_4) for 60 min at 30ºC. 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-^{32}P] ATP, 100 μM ATP, and 10 mM $MgCl_2$ 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 28ºC 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 28ºC, and harvested at a density of 3-5 x 10^7 cells/ml. The cells were washed twice with sporulation medium (SPM; 1% potassium acetate), resuspended in the same medium to 1.5 x 10^7 cells/ml, and further incubated at 28ºC. The formation of asci was assessed by phase-contrast microscopy.

1.3.8. RNA Binding, Immunoprecipitation, and mRNA Detection

Exponentially growing cells (4 x 10^8) were disrupted with glass beads in 200 μl of

extraction buffer (50 mM Tris-HCl (pH 7.5), 150 mM KCl, and 2 mM $MgCl_2$) containing 40 units/μl RNasin (Qiagen), phosphatase inhibitors (10 mM NaF and 1 mM Na_3VO_4), 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 $MgCl_2$) 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 Δ*hog1* Cells

The strain lacking *HOG1* shows severe growth defect, which is accompanied by a G_2 delay and polarized bud formation, on medium containing high Ca^{2+} due to the activation of the cellular Ca^{2+}-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 Ca^{2+}, we screened for suppressor mutants of the Ca^{2+}-sensitive phenotypes of *hog1* deletion cells. The isolated mutants were classified into 6 loci designated *sgh1-sgh6* (for suppressors of Ca^{2+}-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 $CaCl_2$ sensitivity of the Δ*hog1*, 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 Δ*hog1* 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 Δ*bcy1* 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 Δ*bcy1* 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 Δ*bcy1* 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 Δ*bcy1* 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 (Δ*whi3*), 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 G_1/S Transition by Modulating *CLN2* Transcription

As the *whi3* deletion promotes the G_1/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 G_1 phase. The Whi3-S568D mutation, like the deletion one (Δ*whi3*), led to a shortened G_1 phase compared to the length of the G_1 phase in WT cells (Fig. 4A). In contrast, the Whi3-S568A mutation led to a prolonged G_1 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 G_1 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 Δ*whi3* 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 G_1/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 G_1 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 Δ*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 (Δ*bcy1* 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 Δ*bcy1* mutation background (see Δ*bcy1* Whi3-S568A mutant), although the binding ability was slightly reduced by the Δ*bcy1* mutation. This result indicates that the Whi3-S568A mutation is epistatic to the Δ*bcy1* 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 G_1/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 G_1/S progression but also the switch to developmental options such as filamentation and meiosis when challenged in G_1 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 idetified, 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 Δ*whi3* one, accelerated the G_1/S transition (Fig. 4). In addition, the Whi3-S568D mutation led to defective in invasive growth and sporulation as clearly as the Δ*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 Δ*hog1* 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 Δ*hog1* cells in a G_1 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 G_1 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 G_1 delay under high-osmolarity conditions. Alternatively, it is possible that Hog1 and Whi3 antagonize the regulation of restart from G_1 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 Ca^{2+}-sensitive phenotypes of Δ*hog1* cells, it is indicated that Whi3 is also involved in Ca^{2+} signaling. I consider the possible relationship between Whi3 and the Ca^{2+} 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; Garí *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 Δ*bcy1* 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.

Table 1 Strains used in Chapter I

Strain	Genotype	Source or Reference
W303-1A	MATa; *trp1, leu2, ade2, ura3, his3, can1-100*	Lab. stock
SOS5	MATa; Δ*hog1::TRP1* [W303-1A]	Shitamukai *et al*., 2004
SHI338	MATa; Δ*hog1::TRP1 sgh4* [W303-1A]	Kobayashi *et al.*, 2008
SHI336	MATa; *sgh4* [W303-1A]	Kobayashi *et al.*, 2008
SHI301	MATa; Δ*whi3::kanMX6* [W303-1A]	this study
SHI305	MATa; Δ*hog1::TRP1* Δ*whi3::kanMX6* [W303-1A]	this study
SHI294	MATa; *WHI3-3HA::kanMX6* [W303-1A]	this study
YMM501	MATa; Δ*bcy1::URA3 WHI3-3HA::kanMX6* [W303-1A]	this study
YMM502	MATa; *WHI3S568A-3HA::kanMX6* [W303-1A]	this study
YMM504	MATa; Δ*bcy1::URA3 WHI3S568A-3HA::kanMX6* [W303-1A]	this study
YMM505	MATa; *WHI3S568D-3HA::kanMX6* [W303-1A]	this study
YRT73	MATa; Δ*cln3:: HIS3 WHI3-3HA::kanMX6* [W303-1A]	this study
YRT76	MATa; Δ*cln3:: HIS3 WHI3S568A-3HA::kanMX6* [W303-1A]	this study
YRT75	MATa; Δ*cln3:: HIS3 WHI3S568D-3HA::kanMX6* [W303-1A]	this study
YRT74	MATa; Δ*cln3:: HIS3* Δ*whi3::kanMX6* [W303-1A]	this study
W303	MATa/α; *trp1/trp1, leu2/leu2, ade2/ade2, ura3/ura3, his3/his3, can1-100/can1-100*	Lab. stock
YMM507	MATa/α; *WHI3-3HA::kanMX6*/*WHI3-3HA::kanMX6* [W303]	this study
YMM508	MATa/α; *WHI3S568A-3HA::kanMX6*/*WHI3S568A-3HA::kanMX6* [W303]	this study
YMM509	MATa/α; *WHI3S568D-3HA::kanMX6*/*WHI3S568D-3HA::kanMX6* [W303]	this study
YMM510	MATa/α; Δ*whi3::kanMX6*/Δ*whi3::kanMX6* [W303]	this study
YRT91	MATa/α; Δ*cln3::HIS3*/Δ*cln3::HIS3 WHI3-3HA::kanMX6/WHI3-3HA::kanMX6* [W303]	this study
YRT94	MATa/α; Δ*cln3::HIS3*/Δ*cln3::HIS3 WHI3S568A-3HA::kanMX6/WHI3S568A-3HA::kanMX6* [W303]	this study
YRT93	MATa/α; Δ*cln3::HIS3*/Δ*cln3::HIS3 WHI3S568D-3HA::kanMX6/WHI3S568D-3HA::kanMX6* [W303]	this study
YRT92	MATa/α; Δ*cln3::HIS3*/Δ*cln3::HIS3 Δwhi3::kanMX6*/Δ*whi3::kanMX6* [W303]	this study
MLY41a	MATa; *ura3-52*	Lorenz and Heitman 1997
YMM515	MATa; *WHI3S568A-3HA::kanMX6* [MLY41a]	this study
YMM516	MATa; *WHI3S568D-3HA::kanMX6* [MLY41a]	this study
YMM517	MATa; *WHI3-3HA::kanMX6* [MLY41a]	this study
YRT85	MATa; Δ*cln3::URA3* [MLY41a]	this study
YRT87	MATa; Δ*cln3::URA3 WHI3S568A-3HA::kanMX6* [MLY41a]	this study
YRT88	MATa; Δ*cln3::URA3 WHI3S568D-3HA::kanMX6* [MLY41a]	this study
YRT86	MATa; Δ*cln3::URA3 WHI3-3HA::kanMX6* [MLY41a]	this study

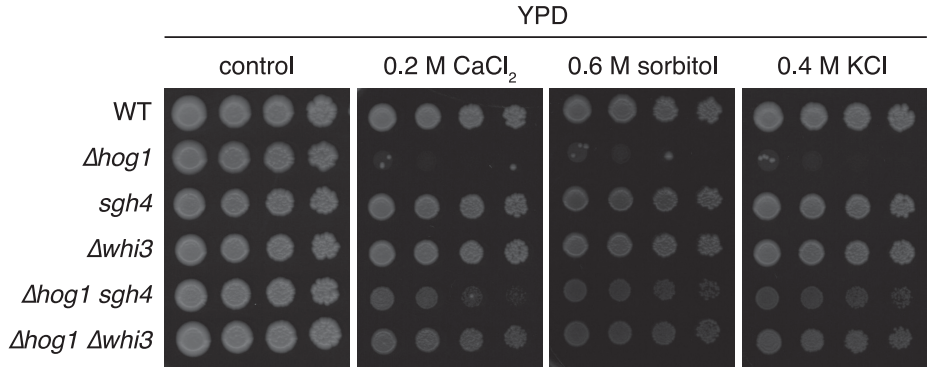

Figure 1 The deletion of the *WHI3* gene suppresses Ca^{2+}- and osmo-sensitivity of Δ*hog1* cells.

Effect of the deletion of the *WHI3* gene on the growth of the Δ*hog1* mutant strain on solid medium. WT, Δ*hog1*, *sgh4*, Δ*whi3*, Δ*hog1 sgh4*, Δ*whi3*, and Δ*hog1* Δ*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.

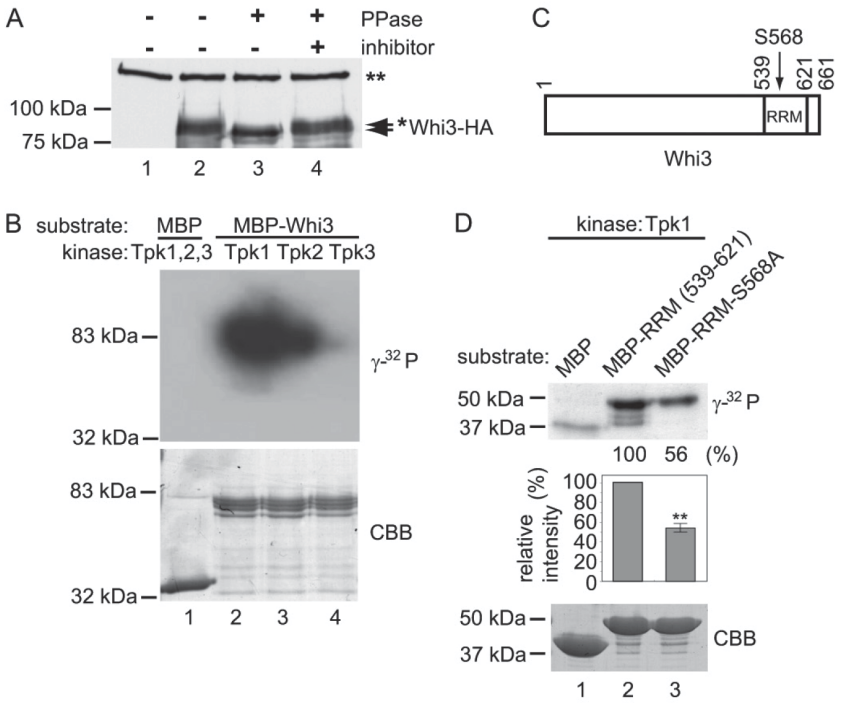
A
PPase
inhibitor
**
100 kDa
75 kDa
*Whi3-HA
1 2 3 4
C
S568
539
621
661
1
RRM
Whi3
B
substrate: MBP MBP-Whi3
kinase: Tpk1,2,3 Tpk1 Tpk2 Tpk3
83 kDa
32 kDa
γ-32 P
CBB
1 2 3 4
D
kinase: Tpk1
substrate: MBP MBP-RRM (539-621) MBP-RRM-S568A
50 kDa
37 kDa
γ-32 P
100 56 (%)
relative intensity (%)
100
80
60
40
20
0
**
CBB
1 2 3
E
Whi3-HA Whi3-S568A-HA
WT Δbcy1 WT Δbcy1
83 kDa
32 kDa
Whi3-HA
Cdc28
1 2 3 4
F
Whi3-HA
WT Δbcy1 WT Δbcy1 WT Δbcy1
PPase
inhibitor
***
100 kDa
75 kDa
**
*
Whi3-HA
1 2 3 4 5 6 7

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 (*PPase*) 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 [γ-^{32}P]ATP (γ-^{32}P). Phosphorylated Whi3 was separated by SDS-PAGE and detected by autoradiography (*upper panel*). MBP and MBP-Whi3 proteins were purified from *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 [γ-^{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 *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 Δ*bcy1* cells. WT and Δ*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 Δ*bcy1* cells expressing Whi3-HA (*lanes 2–7*) were incubated with (+) or without (-) λ-phosphatase or a phosphatase inhibitor. *Lane 1* is a negative control lacking the HA tag on Whi3. After separation by SDSPAGE, Whi3-HA was detected by immunoblotting. *Arrows* indicate the hyperphosphorylated (**), phosphorylated (*), and dephosphorylated forms of Whi3-HA protein. A nonspecific band is indicated (***).

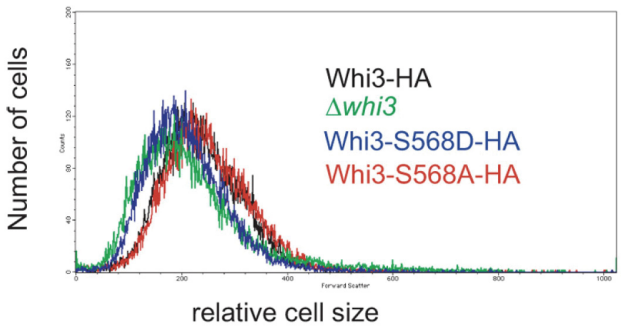

Figure 3 PKA controls cell size by phosphorylating Whi3.

(A) Whi3-HA, Δ*whi3*, 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 10^4 cells.

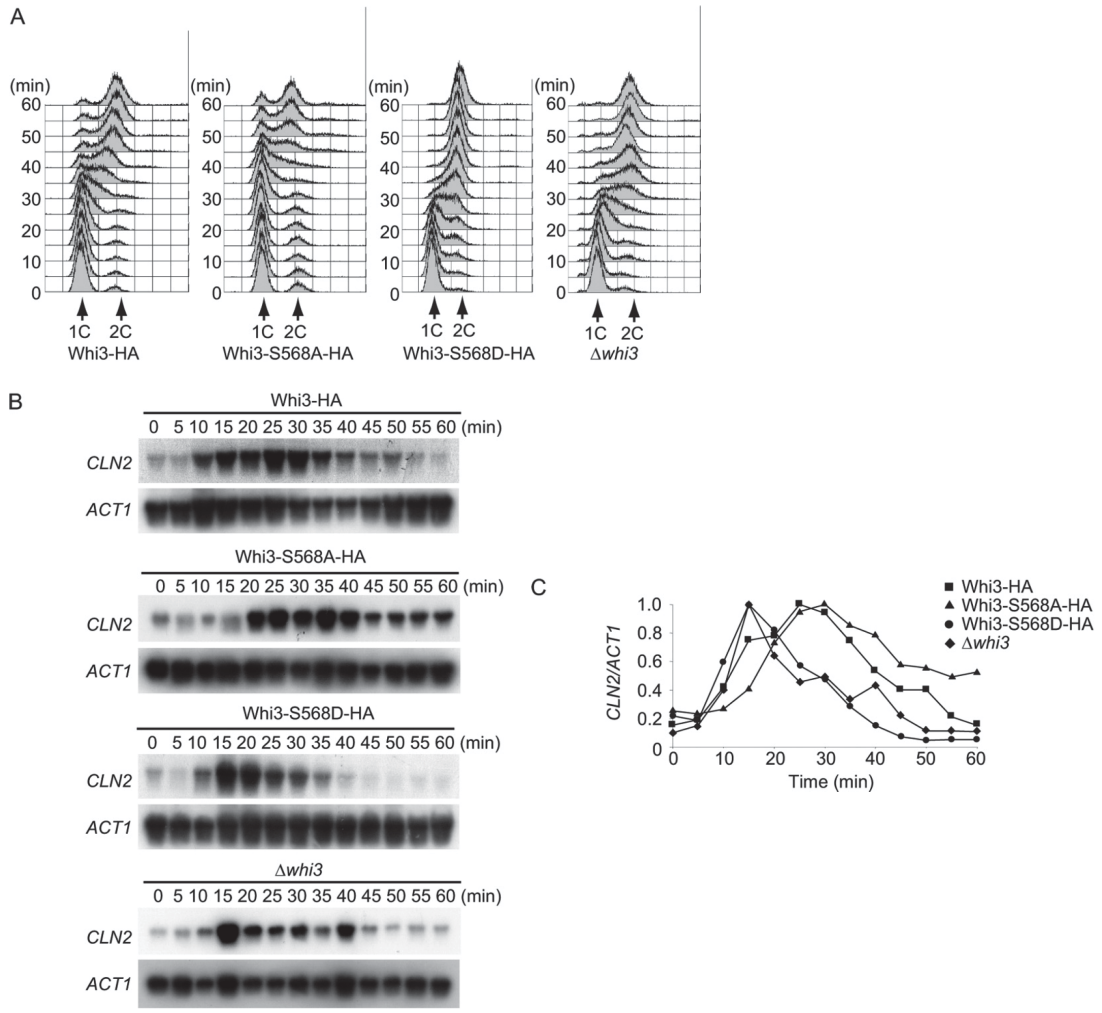

Figure 4　Phosphorylation state of Whi3 at its Ser568 by PKA affects the timing of CLN2 transcription and cell-cycle progression.

(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.

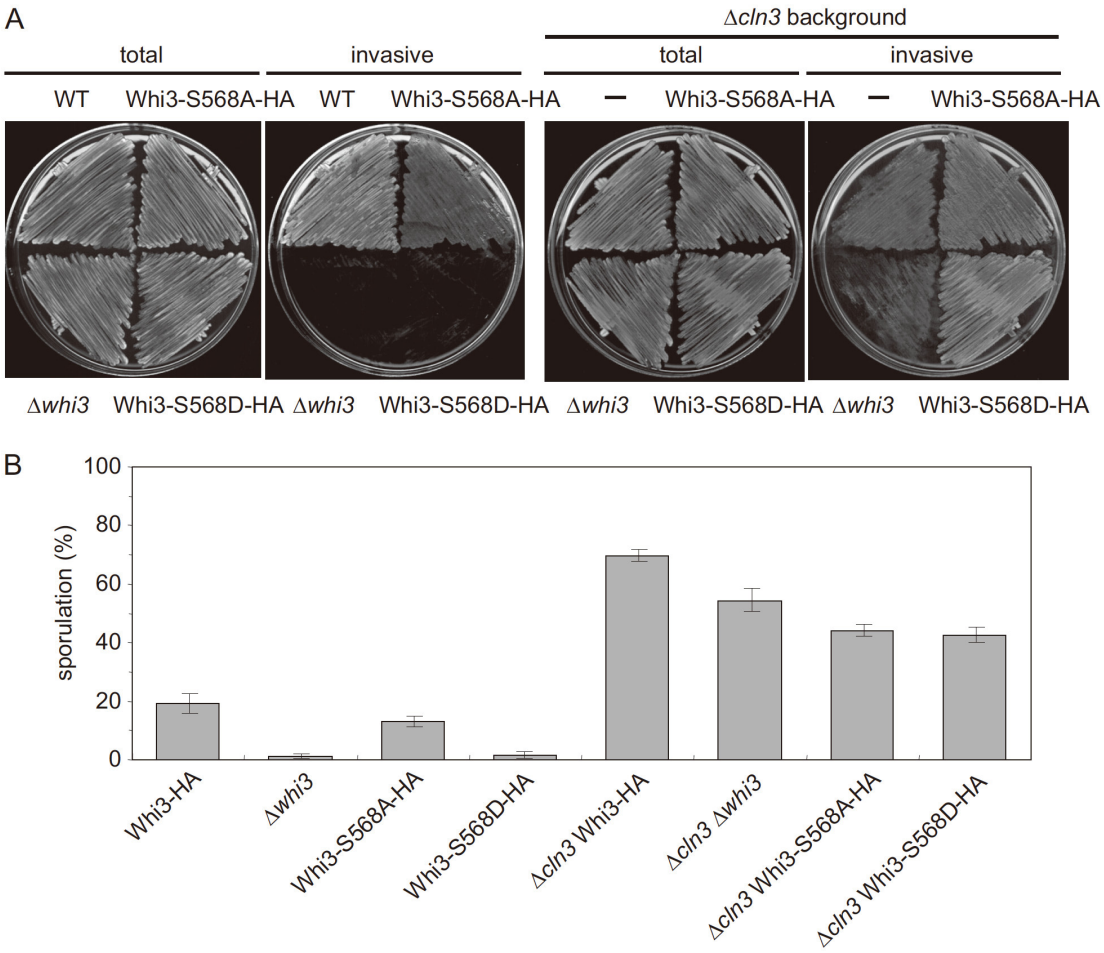

Figure 5 Phosphorylation state of Whi3-Ser568 is important for the growth regulation to switch to or not to switch to meiosis and invasive cell growth.

(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, *Δwhi3*, *Δcln3*, *Δcln3* Whi3-S568A-HA, *Δcln3* Whi3-S568D-HA or *Δcln3 Δwhi3* 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.

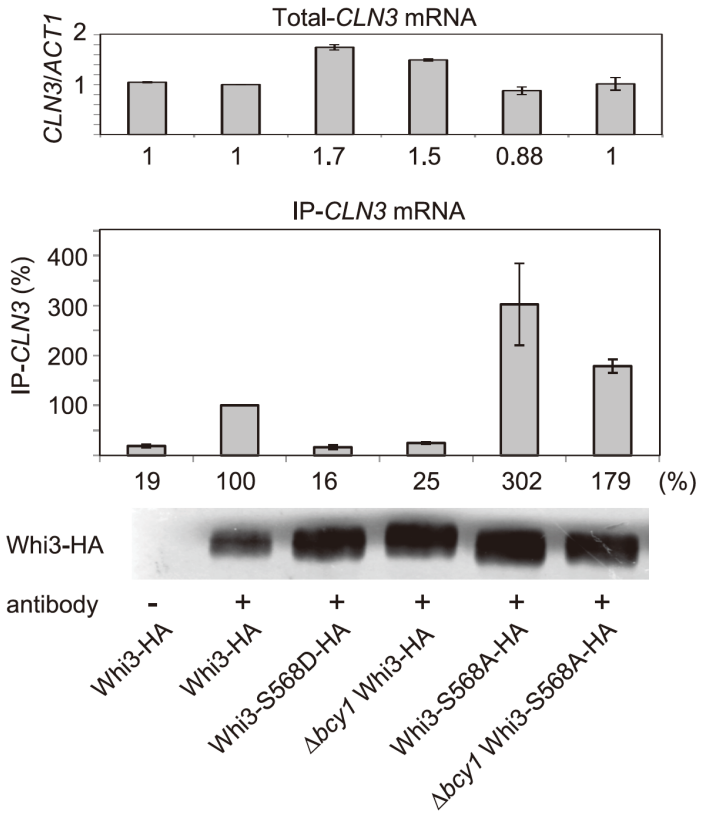

Figure 6 Phosphorylation of Whi3 by PKA inhibits binding of Whi3 to *CLN3* mRNA *in vivo*.

Extracts of cells expressing Whi3-HA, Whi3-S568D-HA, Whi3-S568A-HA, Δ*bcy1* Whi3-HA, or Δ*bcy1* 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 *CLN3* mRNA was normalized to that of *ACT1* mRNA (Total). Values relative to the *CLN3* mRNA level of Whi3-HA (+) are indicated below each bar. Data represent the means ± S.E. of three independent experiments.

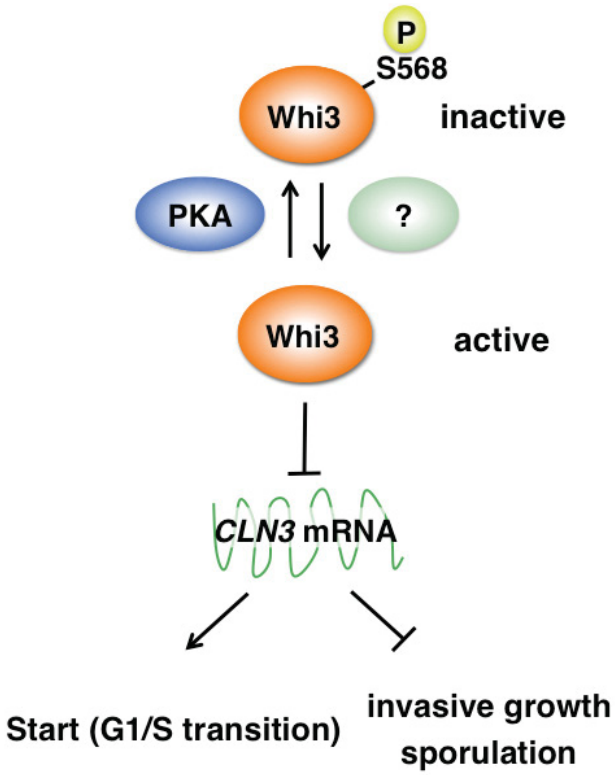

Figure 7　Model for the role of PKA in regulating multiple cellular functions by phosphorylating Whi3 at Ser-568.

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*Δ 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 calucineurin 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*Δ 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*Δ 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 α-klotho, which was originally identified as a putative age-suppressing gene in mice, is involved in the regulatory

system of Ca^{2+} metabolism, suggesting a relationship between Ca^{2+} and aging (Imura *et al*., 2007).

In the budding yeast *Saccharomyces cerevisiae*, the Ca^{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_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^{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^+-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 $CaCl_2$ were delayed in the G_2 phase and showed polarized bud growth, due to the activation of cellular Ca^{2+} signaling pathways in budding yeast (Nakamura *et al*., 1996; Mizunuma *et al*., 1998). Two Ca^{2+} activated pathways, namely, the Mpk1 MAP kinase cascade and the calcineurin pathway, coordinately regulate the G_2/M transition. To identify novel signaling components of the cell cycle and the growth control mediated by Ca^{2+} signaling, we screened for suppressor mutants of the Ca^{2+}-sensitive phenotypes of *zds1* deletion cells and they had been classified into 14 genetic complementation groups (designated *scz1* to *scz14* for suppressor of Ca^{2+}-induced abnormalities of the Δ*zds1* strain). Of these mutants, we identified the mutant allele of *SIR3* as *scz14*. In this chapter, I investigated the relationship between Ca^{2+} signaling and replicative life span (RLS) in yeast. I propose that Ca^{2+} homeostasis/Ca^{2+} 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 Δ*sir3*, Δ*pmc1*, and Δ*pmr1* strains on a W303 background were constructed by gene replacement. Genomic DNA was isolated from the Δ*sir3::kanMX4,* Δ*pmc1::kanMX4,* and Δ*pmr1::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 28ºC 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 28ºC 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 Δ*zds1* Cells

ZDS1 null mutant (Δ*zds1*) 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 Δ*zds1* 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 Δ*zds1* 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 Δ*sir3* cells in further experiments.

Why did the Δ*sir3* mutation suppress only the Ca^{2+}-induced hyperpolarized bud growth but not the G_2-delay of Δ*zds1* cells? Because the Ca^{2+} signal induced the

polarized bud growth by elevating the level of G_1-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 Δ*zds1* cells were decreased by the disruption of *SIR3* (compare Δ*zds1* and Δ*zds1* Δ*sir3* in Fig. 8D), suggesting that the suppression of the Ca^{2+} sensitive phenotype of the Δ*zds1* cells by the *sir3* deletion was caused by a defect in the elevation of the Cln2 level in response to Ca^{2+}. Additionally, similar effect of the *sir3* disruption on the Cln2 level was observed on wild-type (WT) background (compare WT and Δ*sir3* in Fig. 8D). These results indicate that Sir3 is important for the elevation of the Cln2 level in the presence and absence of external Ca^{2+}. 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 Ca^{2+}-signaling and lifespan.

2.4.2 Increase in External and Intracellular Ca^{2+} Levels Causes Shortened RLS

I first investigated whether the extracellular Ca^{2+} 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 $CaCl_2$ showed a decreased RLS relative to normal medium (Fig. 9A). To check the specificity of Ca^{2+} on the yeast RLS, I examined the effect of $MgCl_2$ and KCl on 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 (Nakamura *et al*., 1993; Farcasanu *et al*., 1995). As the result, neither $MgCl_2$ (100 mM) nor KCl (150 mM) altered significantly the RLS of WT cells (Fig. 9A), indicating that external Ca^{2+} reduced RLS.

To investigate the possibility that extracellular Ca^{2+} shortens RLS by activating intracellular Ca^{2+} signaling, I measured the RLS of Δ*zds1* cells, in which Ca^{2+}-signaling pathways might be activated by increase in internal Ca^{2+} level (Mizunuma *et al*., 1998). As expected, the mean RLS of Δ*zds1* cells was shorter than that of WT cells under the normal condition (Fig. 9B).

To examine whether the *zds1* deletion affected the activation of Ca^{2+}-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 *zds1*Δ 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 (Δ*cnb1*) (Fig. 9C). As expected, β-galactosidase activity in the Δ*zds1* 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 Δ*zds1* and Δ*zds1* Δ*cnb1*). These results suggested that the increase in cellular Ca^{2+} level in the Δ*zds1* 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 *zds1*Δ 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 Δ*pmr1* and Δ*pmc1* cells. The Δ*pmr1* cells but not the Δ*pmc1* 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 Δ*pmr1* cells, but not in

the Δ*pmc1*, 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 Δ*zds1* 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 Δ*zds1* cells. As expected, Δ*zds1* Δ*cnb1* double deletion cells displayed a longer RLS compared to Δ*zds1* single deletion cells (Fig. 10B). Interestingly, the mean RLS of Δ*zds1* Δ*cnb1* 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 Δ*zds1* cells.

2.4.6 Calcineurin and Sir3 Act Redundantly in the Regulation of RLS

I next examined the effect of the calcineurin deletion (Δ*cnb1*) 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 Δ*cnb1* Δ*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 Δ*cnb1* Δ*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 Δ*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 Δ*zds1* cells were suppressed by the calcineurin deletion mutations (Mizunuma *et al*., 1998). Moreover, calcineurin activity was enhanced in the Δ*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 Δ*pmr1* cells show a severe reduction in mean RLS compared to other cells (Δ*zds1*, Δ*cnb1*, and Δ*sir3*)? 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 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 H_1-ATPases, reduced RLS (Kaeberlein *et al*., 2005). These results suggest that the severe RLS reduction of Δ*pmr1* cells was derived not only from altered Ca^{2+} 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 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 such as human, and whether the corresponding pathways are a part of a conserved network involved in the control of biological aging.

Table2 Strains used in Chapter II

Strain	Genotype	Source or Reference
W303-1A	MATa; *trp1, leu2, ade2, ura3, his3, can1-100*	Lab. stock
YAT1	MATa; Δ*zds1::TRP1* [W303-1A]	Mizunuma *et al.*, 1998
YJY10	Δ*zds1::TRP1* Δ*sir3::kanMX4* [W303-1A]	this study
YJY9	Δ*sir3::kanMX4* [W303-1A]	this study
YGA13	MATa; Δ*pmc1::kanMX4* [W303-1A]	this study
YGA14	MATa; Δ*pmr1::kanMX4* [W303-1A]	this study
DTH14	MATa; Δ*cnb1::HIS3* [W303-1A]	Nakamura *et al.*, 1993
YMM2	MATa; Δ*zds1::TRP1* Δ*cnb1::HIS3* [W303-1A]	Mizunuma *et al.*, 1998
YJY12	Δ*cnb1::HIS3* Δ*sir3::kanMX4* [W303-1A]	this study

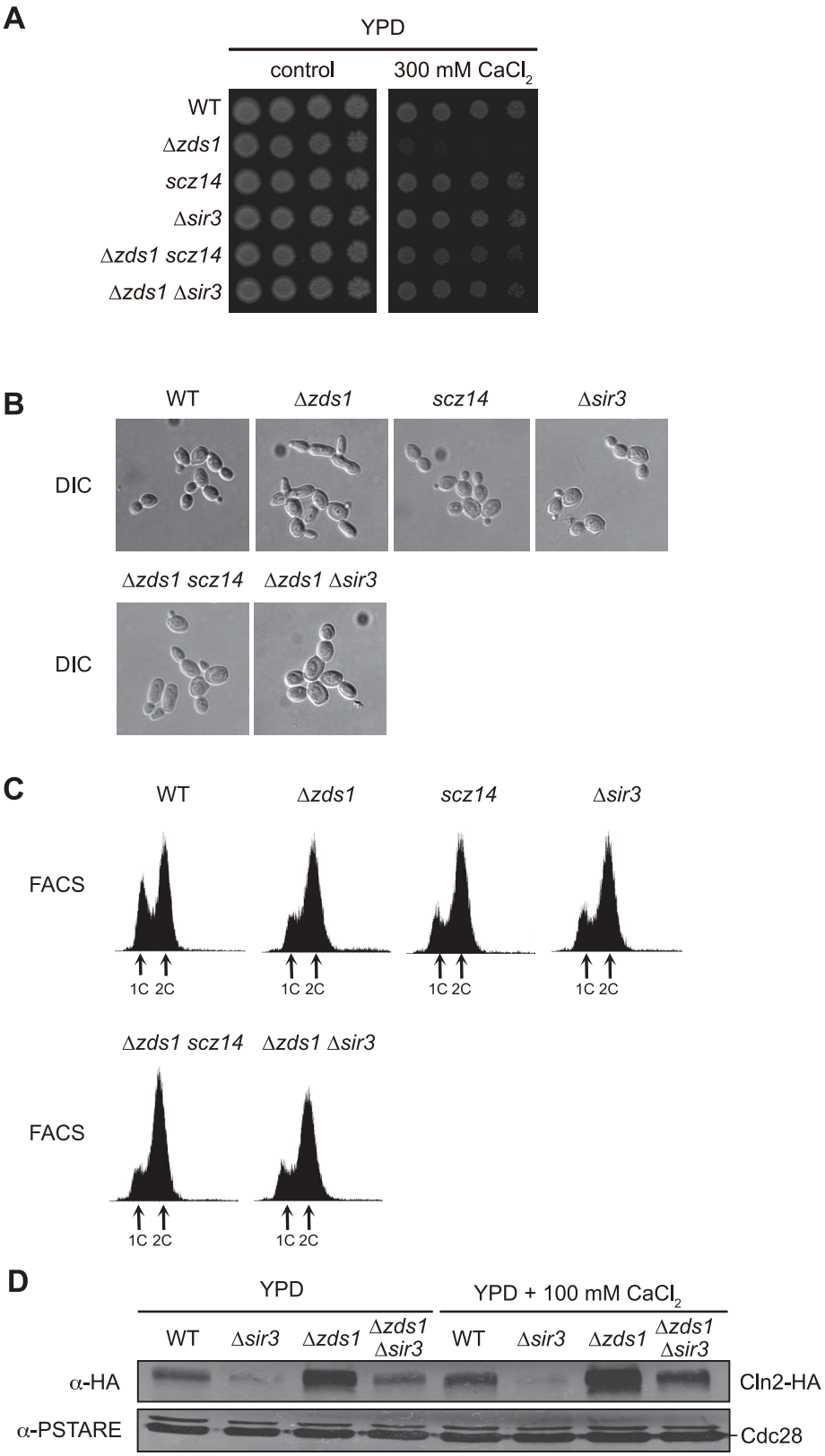
A
YPD
control
300 mM CaCl2
WT
Δzds1
scz14
Δsir3
Δzds1 scz14
Δzds1 Δsir3
B
WT
Δzds1
scz14
Δsir3
DIC
Δzds1 scz14
Δzds1 Δsir3
DIC
C
WT
Δzds1
scz14
Δsir3
FACS
1C 2C
Δzds1 scz14
Δzds1 Δsir3
FACS
1C 2C
D
YPD
YPD + 100 mM CaCl2
WT
Δsir3
Δzds1
Δzds1 Δsir3
WT
Δsir3
Δzds1
Δzds1 Δsir3
α-HA
Cln2-HA
α-PSTARE
Cdc28

Figure 8 The deletion of *SIR3* gene suppresses various phenotypes of the Δ*zds1* strain.

(A) Effect of the deletion of *SIR3* gene on the growth of the Δ*zds1* mutant strain on solid medium. WT, Δ*zds1*, *scz14*, Δ*sir3*, Δ*zds1 scz14*, and Δ*zds1* Δ*sir3* cells were spotted on YPD solid medium containing 300 mM $CaCl_2$, 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 $CaCl_2$ at 25ºC (B). Flow cytometry analysis (FACS) of PI-stained cells of various strains after 6 h of incubation with 100 mM $CaCl_2$ at 25ºC (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, Δ*zds1*, Δ*sir3*, and Δ*zds1* Δ*sir3* strain were suspended in YPD containing 100 mM $CaCl_2$, 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.

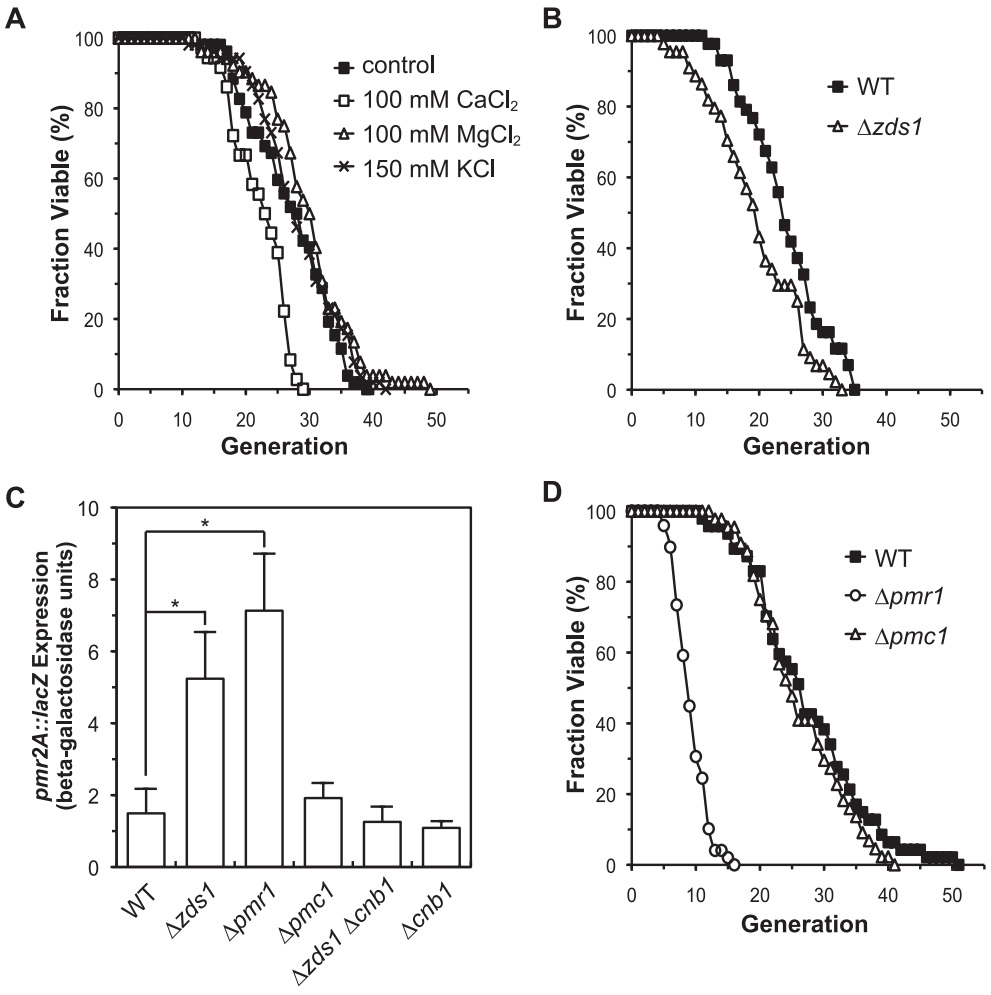

Figure 9 An increase in Ca^{2+} levels led to reduced RLS.

(A) RLS analysis of WT cells grown on YPD (control), YPD plus 100 mM $CaCl_2$, YPD plus 100 mM $MgCl_2$, and YPD plus 150 mM KCl at 25ºC. Mean RLS in terms of numbers of cell divisions: control, 27.4 (n = 52); 100 mM $CaCl_2$, 22.5 (n = 36); 100 mM $MgCl_2$, 29.8 (n = 52); 150 mM KCl, 28.3 (n = 52). (B) RLS analysis of WT and Δ*zds1* mutant cells grown on YPD at 25ºC. Mean RLSs: WT, 24.3 (n = 43); Δ*zds1*, 19.6 (n = 44). (C) Expression of the CDRE-dependent reporter gene in WT, Δ*zds1*, Δ*zds1* Δ*cnb1*, Δ*pmc1*, Δ*pmr1,* and Δ*cnb1* mutant cells. *P<0.001 (D) RLS analysis of WT, Δ*pmc1*, and Δ*pmr1* mutant cells grown on YPD at 25ºC. Mean RLSs: WT, 27.5 (n = 47); Δ*pmc1*, 26.3 (n = 44); Δ*pmr1*, 9.4 (n =49).

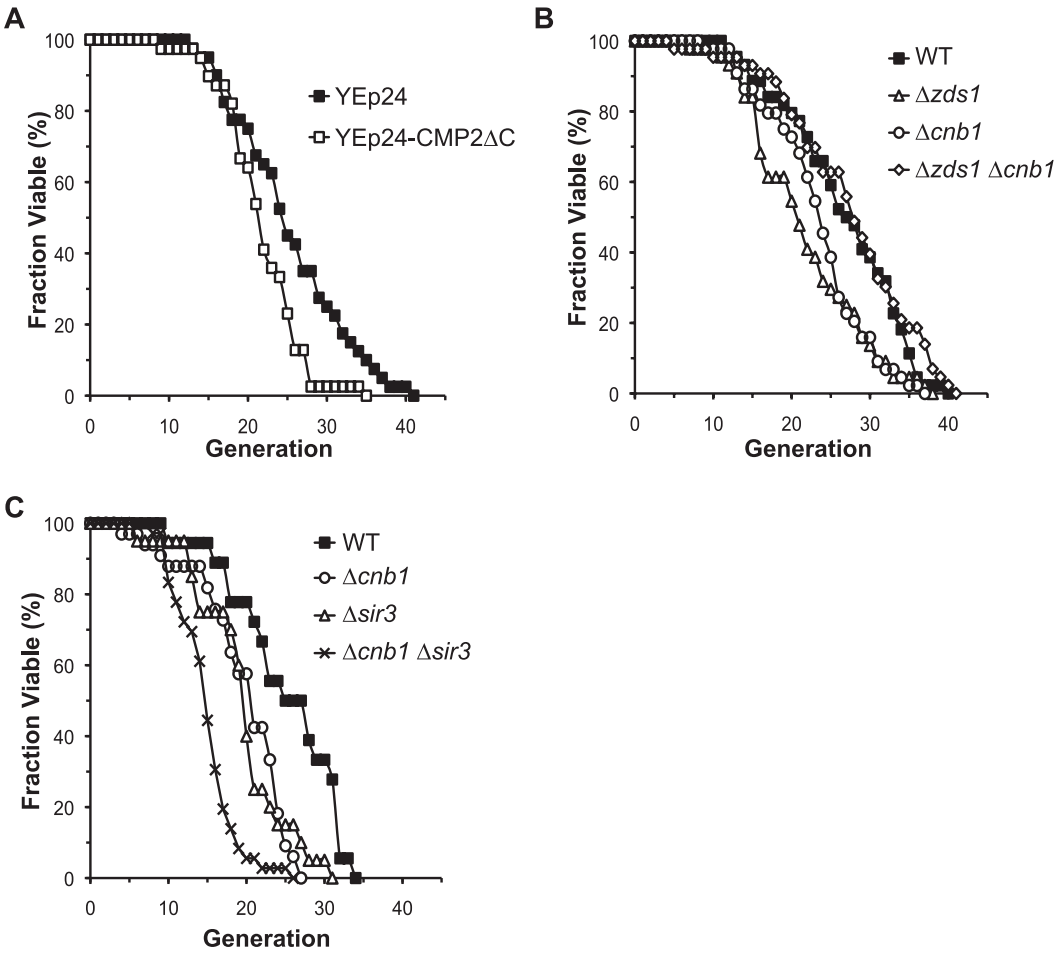

Figure 10 Effect of over-expression and deletion of calcineurin on RLS.

(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, Δ*zds1*, Δ*cnb1*, and Δ*zds1* Δ*cnb1* mutant cells grown on YPD at 25ºC. Mean RLSs: WT, 26.9 (n =44); Δ*zds1*, 21.8 (n = 44); Δ*cnb1*, 23.5 (n = 44); Δ*zds1* Δ*cnb1*, 27.6 (n = 43). (C) RLS analysis of WT, Δ*cnb1*, Δ*sir3*, and Δ*cnb1* Δ*sir3* mutant cells grown on YPD at 25ºC. Mean RLSs: WT, 25.2 (n = 18); Δ*cnb1*, 19.7 (n = 33); Δ*sir3*, 19.6 (n = 20); Δ*cnb1* Δ*sir3*, 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 G_1/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 Ca^{2+} affected the replicative life span (RLS) of yeast. I described that increased external and intracellular Ca^{2+} levels caused decreased RLS. Consistently, the deletion of *ZDS1* gene, which showed the increased cellular Ca^{2+} level, and the activation of calcineurin led to a shortened RLS. Further, the shortened RLS of Δ*zds1* cells was suppressed by the calcineurin deletion. Moreover, I demonstrated that the strain lacking *CNB1* gene had the deceased RLS, suggesting that the basal activity of calcineurin is essential for longevity in yeast. Thus, our results indicated that Ca^{2+} homeostasis/ Ca^{2+} signaling are required to regulate longevity in budding yeast. We also reported that other *SCZ* genes (a suppressor mutation of the Ca^{2+}-sensitive phenotypes of Δ*zds1* cells) were involved in the regulation of yeast life span (Tsubakiyama *et al*., 2011). Since the calcium ion (Ca^{2+}) is a universal second messenger in various organisms, it will be very interesting to investigate whether the collapse of Ca^{2+} 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 Ca^{2+} homeostasis/ Ca^{2+} 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 Ca^{2+}-sensitive phenotypes of Δ*hog1* cells, it is expected that Whi3 is also involved in Ca^{2+} signaling. How dose Whi3 function in Ca^{2+} signaling pathways? Our recent study indicated that Whi3 acted as a

negative regulator for restart from G_1 arrest under osmotic stress (Mizunuma *et al*., 2013a). Moreover, it was revealed that cells showed transient G_1 arrest under high Ca^{2+} conditions, and that high Ca^{2+} 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 G_1 arrest under high Ca^{2+} conditions as well as osmotic stress. We previously reported that the Ca^{2+} 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 Ca^{2+} 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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Ras/cAMP-dependent Protein Kinase (PKA) Regulates Multiple Aspects of Cellular Events by Phosphorylating the Whi3 Cell Cycle Regulator in Budding Yeast*

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Background: Whi3 is known as a negative regulator of the G_1 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.

The Start/G_1 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 G_1 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 led to its decreased interaction with *CLN3* G_1 cyclin mRNA and was required for the promotion of G_1/S progression. Furthermore, we demonstrate that the phosphomimetic S568D mutation of Whi3 prevented the developmental fate switch to sporulation or invasive growth. Thus, PKA modulated the function of Whi3 by phosphorylation, thus implicating PKA-mediated modulation of Whi3 in multiple cellular events.

Eukaryotic cells monitor internal and external signals to commit themselves to cell division or to a switch to alternative developmental fates during the G_1 phase. In the budding yeast *Saccharomyces cerevisiae*, this control network is called Start (1). Start is initiated by the G_1 cyclin Cln3, which is associated with the cyclin-dependent kinase Cdc28 (2).

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 G_1 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 G_1 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 G_1/S transition. It has long been thought that the G_1 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-

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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 G_1/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 (*MAT***a** *trp1-1 leu2-3 ade2-1 ura3-1 his3-11 can1-100*), SHI301 (*MAT***a** Δ*whi3*::*kanMX6*), SHI294 (*MAT***a** *WHI3-3HA*::*kanMX6*), YMM501 (*MAT***a** Δ*bcy1*::*URA3 WHI3-3HA*::*kanMX6*), YMM502 (*MAT***a** *WHI3-S568A-3HA*::*kanMX6*), YMM504 (*MAT***a** Δ*bcy1*::*URA3 WHI3-S568A-3HA*::*kanMX6*), YMM505 (*MAT***a** *WHI3-S568D-3HA*::*kanMX6*), YRT73 (*MAT***a** Δ*cln3*::*HIS3 WHI3-3HA*::*kanMX6*), YRT76 (*MAT***a** Δ*cln3*::*HIS3 WHI3-S568A-3HA*::*kanMX6*), YRT75 (*MAT***a** Δ*cln3*::*HIS3 WHI3-S568D-3HA*::*kanMX6*), YRT74 (*MAT***a** Δ*cln3*::*HIS3* Δ*whi3*::*kanMX6*), YTO1 (*MAT***a** *HIS3 GAL1-WHI3-3HA*::*kanMX6*), YTO2 (*MAT***a** *HIS3 GAL1-WHI3-S568D-3HA*::*kanMX6*), YMM507 (*MAT***a**/α *WHI3-3HA*::*kanMX6/WHI3-3HA*::*kanMX6*), YMM508 (*MAT***a**/α *WHI3-S568A-3HA*::*kanMX6/WHI3-S568A-3HA*::*kanMX6*), YMM509 (*MAT***a**/α *WHI3-S568D-3HA*::*kanMX6/WHI3-S568D-3HA*::*kanMX6*), YMM510 (*MAT***a**/α Δ*whi3*::*kanMX6/*Δ*whi3*::*kanMX6*), YRT91 (*MAT***a**/α Δ*cln3*::*HIS3 WHI3-3HA*::*kanMX6/*Δ*cln3*::*HIS3 WHI3-3HA*::*kanMX6*), YRT94 (*MAT***a**/α Δ*cln3*::*HIS3 WHI3-S568A-3HA*::*kanMX6/*Δ*cln3*::*HIS3 WHI3-S568A-3HA*::*kanMX6*), YRT93 (*MAT***a**/α Δ*cln3*::*HIS3 WHI3-S568D-3HA*::*kanMX6/*Δ*cln3*::*HIS3 WHI3-S568D-3HA*::*kanMX6*), and YRT92 (*MAT***a**/α Δ*cln3*::*HIS3* Δ*whi3*::*kanMX6/*Δ*cln3*::*HIS3* Δ*whi3*::*kanMX6*), all derivatives of strain W303-1A. Also used were the following derivatives of the Σ strain: MLY41a (*MAT***a** *ura3-52*), YMM515 (*WHI3-S568A-3HA*::*kanMX6* in MLY41a), YMM516 (*WHI3-S568D-3HA*::*kanMX6* in MLY41a), YMM517 (Δ*whi3*::*kanMX6* in MLY41a), YRT85 (Δ*cln3*::*URA3 ura3-52* in MLY41a), YMRT87 (Δ*cln3*::*URA3 WHI3-S568A-3HA*::*kanMX6* in MLY41a), YRT88 (Δ*cln3*::*URA3 WHI3-S568D-3HA*::*kanMX6* in MLY41a), and YRT86 (Δ*cln3*::*URA3* Δ*whi3*::*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 SalI, and then cloned into the BamHI- and SalI-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 SalI, and then cloned into the BamHI- and SalI-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-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 3-HA 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 ApaI-SphI fragment of the pT-Whi3–5T-3HA plasmid was cloned into the ApaI- and SphI-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 Δ*whi3* 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 ΣMLY41a 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-HA 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 $MnCl_2$ with or without phosphatase inhibitors (50 mM NaF and 5 mM sodium orthovanadate) for 60 min at 30 °C. After the phosphatase treatment, 4× 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-

[4] The abbreviations used are: MBP, maltose-binding protein; RRM, RNA recognition motif; YPD, yeast extract/peptone/dextrose.

[5] The oligonucleotide primer sequences used in this study are available upon request.

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-^{32}P]ATP, 100 μM ATP, and 10 mM $MgCl_2$ 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 (ΣMLY41a, 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 $\times$ 10^7 cells/ml. The cells were washed twice with sporulation medium (1% potassium acetate), resuspended at 1.5 $\times$ 10^7 cells/ml in the same medium, and further incubated at 28 °C. The formation of asci was assessed by phase-contrast microscopy.

RNA Binding, Immunoprecipitation, and mRNA Detection—Exponentially growing cells (4 $\times$ 10^8) were disrupted with glass beads in 200 μl of extraction buffer (50 mM Tris-HCl (pH 7.5), 150 mM KCl, and 2 mM $MgCl_2$) 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 $\times$ *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 $MgCl_2$) 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 G_1 cyclin for cell size control (3) and shown to play an important role in regulating Cln3 activity in G_1 (4). Despite its linkage to multiple cellular events, including cell cycle progression and developmental fate, the upstream regulator(s) of Whi3 has still not 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. 1*A*, *lane 2*). The phosphatase-treated Whi3-HA protein migrated faster

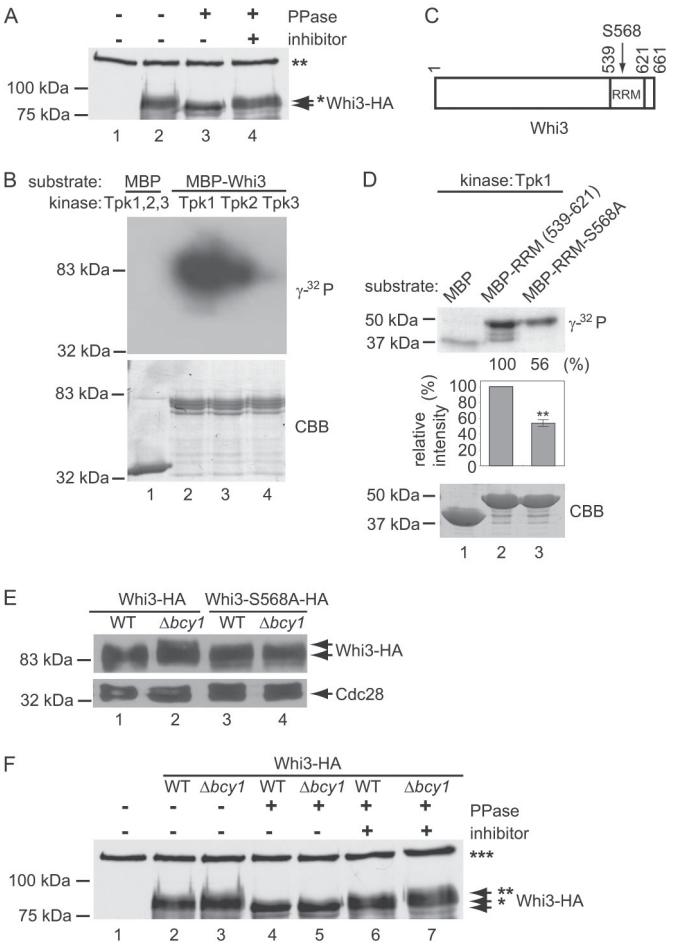

FIGURE 1. **Whi3 is phosphorylated by PKA *in vitro* and *in vivo*.** *A*, protein phosphatase treatment of Whi3-HA. Whole cell extracts of WT cells expressing Whi3-HA (*lanes 2–4*) were incubated with (+) or without (−) λ-phosphatase (*PPase*) or a phosphatase inhibitor. *Lane 1* is a negative control lacking the HA tag on Whi3. After separation by SDS-PAGE, Whi3-HA was detected by immunoblotting. *Arrows* indicate the phosphorylated (*) and dephosphorylated forms of Whi3-HA protein. A nonspecific band is indicated (**). *B*, recombinant MBP-Whi3 or MBP (control) was incubated with GST-Tpk1, GST-Tpk2, or GST-Tpk3 in the presence of [γ-^{32}P]ATP (γ-^{32}*P*). Phosphorylated Whi3 was separated by SDS-PAGE and detected by autoradiography (*upper panel*). MBP and MBP-Whi3 proteins were purified from *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 [γ-^{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 *E. coli* BL40 and stained with Coomassie Brilliant Blue (*lower panel*). *E*, mobility shift of Whi3-HA or Whi3-S568A-HA from WT and Δ*bcy1* cells. WT and Δ*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 Δ*bcy1* cells expressing Whi3-HA (*lanes 2–7*) were incubated with (+) or without (−) λ-phosphatase or a phosphatase inhibitor. *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 (***).

than the untreated protein (Fig. 1*A*, 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-^{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. 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. 1*B*, *lanes 1* and *2*). The two other isoforms of PKA, *i.e.* Tpk2 and Tpk3, exhibit moderate kinase activity in this assay (Fig. 1*B*, *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. 1*C*). 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. 1*D*), 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. 1*E*, *lanes 1* and *2*). The phosphatase-treated Whi3-HA protein in *bcy1*-deleted cells migrated faster than the untreated protein (Fig. 1*F*, 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. 1*E*). In the WT cells, the phosphorylation level of the Whi3-S568A-HA mutant protein was indistinguishable from that of the Whi3-HA protein (Fig. 1*E*, *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. 1*E*, *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 (Δ*whi3*), was smaller compared with the WT cells (Fig. 2*A*). These results indicate that the phosphorylation of Whi3 by

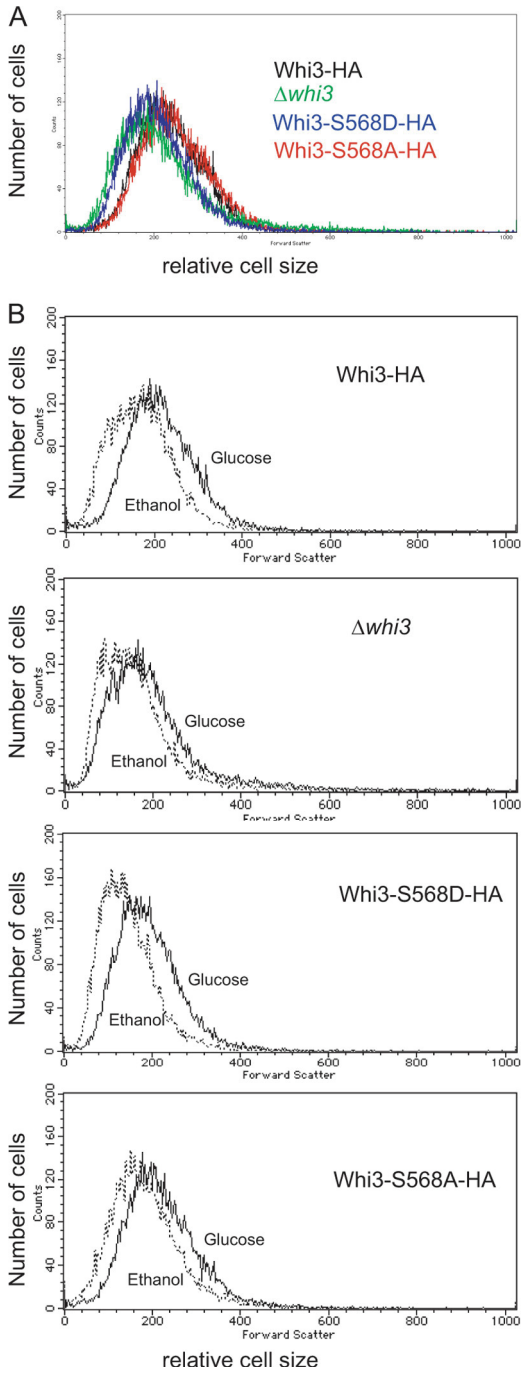

FIGURE 2. **PKA controls cell size by phosphorylating Whi3.** *A*, Whi3-HA, Δ*whi3*, Whi3-S568A-HA, and Whi3-S568D-HA cells were grown in YPD medium. Relative cell size was determined by measuring forward angle light scattering with a FACSCalibur (BD Biosciences). Each histogram was obtained from 2×10^4 cells. *B*, representative size distributions of log-phase cultures of the indicated strains in YP + 2% glucose (*solid lines*) or YP + 2% ethanol (*dotted lines*). Cells were grown in YPD medium to mid-log phase, and cells were re-inoculated into YP + 2% glucose or YP + 2% ethanol and grown to mid-log phase. The cell size of samples was determined using a FACSCalibur.

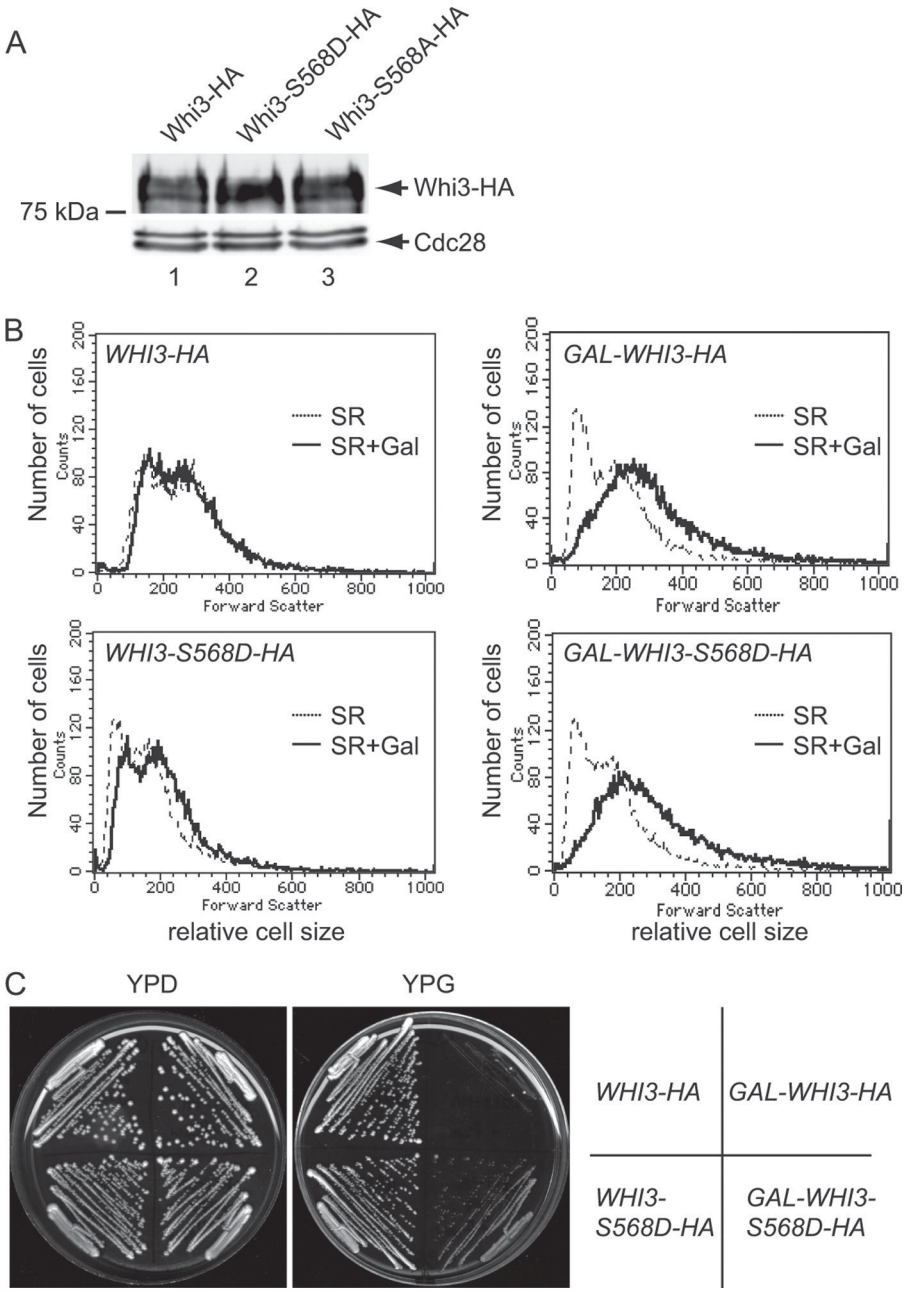

FIGURE 3. **Whi3-S568D-HA is a functional mutation.** *A*, the Whi3 protein level is comparable among mutations. Cells expressing Whi3-HA, Whi3-S568D-HA, or Whi3-S568A-HA were grown in YPD medium to mid-log phase. Proteins of whole cell extracts were separated by SDS-PAGE and detected by immunoblotting with anti-HA (for Whi3-HA, Whi3-S568D-HA, and Whi3-S568A-HA) and anti-PSTAIRE (for Cdc28 as a loading control) antibodies. *B* and *C*, Whi3-S568D-HA is functional. *B*, *WHI3*-HA, *WHI3-S568D*-HA, *GAL-WHI3*-HA, or *GAL-WHI3-S568D*-HA cells in synthetic complete medium (SR−Ura, +2% raffinose medium) were transferred to SR−Ura (+2% raffinose medium, with the *GAL* promoter turned off) or SR+Gal−Ura (+2% galactose medium, with the *GAL* promoter turned on), and the cells were incubated at 28 °C for 8 h, after which cell size was measured. *C*, *WHI3*-HA, *WHI3-S568D*-HA, *GAL-WHI3*-HA, or *GAL-WHI3-S568D*-HA cells were streaked on YP + 2% dextrose or YP + 2% galactose plates.

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 (Whi3-HA) growing in glucose medium was larger than that in ethanol medium (Fig. 2*B*). The cell size of all *whi3* mutant strains (Δ*whi3*, 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. 3*A*). Because phosphomimetic Whi3-S568D-HA mutant cells seemed to behave like the Δ*whi3* 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

purpose, we constructed strains containing a chromosomally integrated gene coding for the *GAL1* promoter upstream of the *WHI3*-HA or *WHI3-S568D*-HA gene. As shown in Fig. 3 (*B* and *C*), wild-type (*WHI3*-HA) and *WHI3-S568D*-HA cells expressing the protein under the control of their own promoter in the presence or absence of galactose in the medium were similar in size and growth. In contrast, the size of cells overexpressing *WHI3-S568D*-HA from the *GAL1* promoter in the presence of galactose, like that of cells overexpressing *WHI3*-HA, was larger (Fig. 3*B*). Furthermore, overexpression of the *WHI3-S568D*-HA mutant caused slow growth but was not lethal (Fig. 3*C*). Therefore, we conclude that the Whi3-S568D mutation is not a null mutation of Whi3.

Phosphorylation State of Ser-568 in Whi3 Affects G_1/S Transition by Modulating CLN2 Transcription—As the *whi3* deletion promotes the G_1/S transition by accelerating *CLN2* transcription (4), we next investigated the role of the PKA-mediated Whi3 phosphorylation in the cell cycle. We examined the cell cycle progression and *CLN2* transcription of Whi3 mutant cells synchronized in the G_1 phase. Like the deletion mutation (Δ*whi3*), the Whi3-S568D mutation led to a shortened G_1 phase compared with the length of the G_1 phase in WT cells (Fig. 4*A*). In contrast, the Whi3-S568A mutation led to a prolonged G_1 phase (Fig. 4*A*). Consistent with the effect of these mutations on cell cycle progression, transcription of the *CLN2* mRNA was accelerated by the Whi3-S568D mutation and decelerated by the Whi3-S568A mutation (Fig. 4, *B* and *C*). These results suggest that the PKA-mediated Whi3 phosphorylation at Ser-568 inhibits Whi3 function, thus promoting the start of the cell cycle.

Phosphorylation of Ser-568 in Whi3 Is Important for Cell Fate Determination—Whi3 is important for restraining Cln3 function in the G_1 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. 5*A*). Furthermore, the homozygous diploid Whi3-S568D-HA mutant, but not the Whi3-S568A-HA mutant, failed to sporulate (Fig. 5*B*). As it has been reported that defects in both sporulation and invasive growth of Δ*whi3* 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 G_1/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. 5, *A* and *B*). These results indicate that the PKA-mediated phosphorylation of Ser-568 in Whi3 is essential for cell fate determination in the G_1 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 Δ*bcy1*

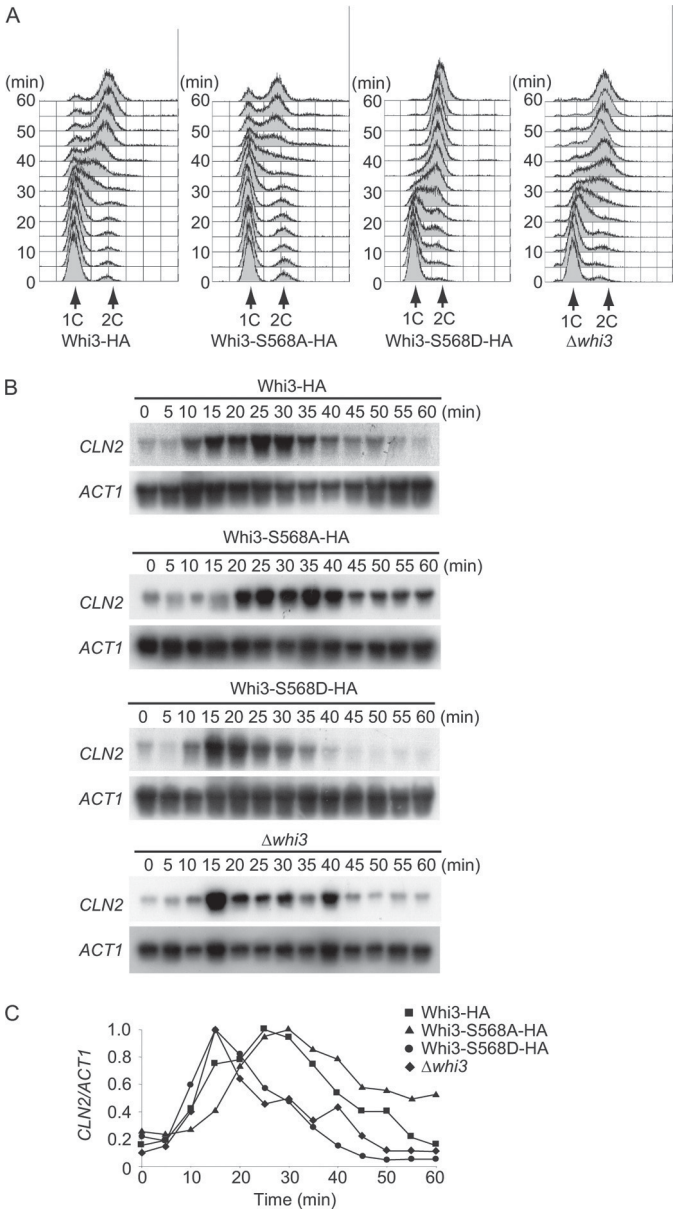

FIGURE 4. **Phosphorylation state of Whi3 at Ser-568 by PKA affects the timing of *CLN2* transcription and cell cycle progression.** *A*, effect of the Whi3-S568A and Whi3-S568D mutations on cell cycle progression. The Whi3-HA, Whi3-S568A-HA, Whi3-S568D-HA, and Δ*whi3* strains were synchronized with 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 *B* was normalized to that of the *ACT1* mRNA. For each strain, the maximum value for the first cell cycle is referred to as 1.

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 (Δ*bcy1* Whi3-HA) forms of Whi3 showed reduced interaction with *CLN3* mRNA in this assay (Fig. 6*A*). Conversely, the Whi3-S568A mutant displayed increased interaction with *CLN3* mRNA (Fig. 6*A*). Moreover, the increase in the ability of Whi3-S568A to bind to *CLN3* mRNA was still observed in the Δ*bcy1* mutation background (see

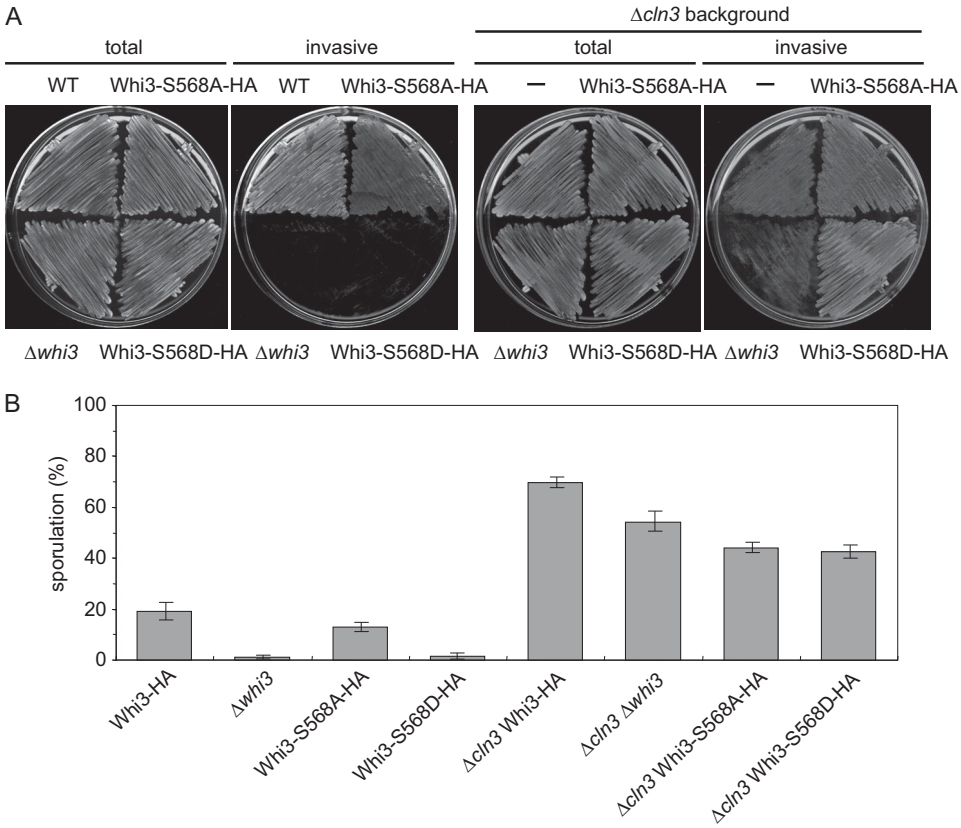

FIGURE 5. **Phosphorylation state of Whi3 Ser-568 is important for growth regulation to switch to or not to switch to meiosis or invasive cell growth.** *A*, effect of the Whi3-S568A or Whi3-S568D mutation on the invasive growth of the haploid Σ1278bMLY41a strain on YPD plates. WT, Whi3-S568A-HA, Whi3-S568D-HA, Δ*whi3*, Δ*cln3*, Δ*cln3* Whi3-S568A-HA, Δ*cln3* Whi3-S568D-HA, or Δ*cln3* Δ*whi3* cells were streaked onto YPD plates and cultured at 28 °C for 2 days. *B*, 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.

Δ*bcy1* Whi3-S568A mutant), although the binding ability was slightly reduced by the Δ*bcy1* mutation. This result indicates that the Whi3-S568A mutation is epistatic to the Δ*bcy1* 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 G_1/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 (*CLN3*^{mGCAU}) in their *CLN3* mRNA due to mutations in the 14 GCAT sites in the *CLN3* gene show a decrease in cell size similar to Δ*whi3* cells (7). If Whi3-S568A binds through GCAU sites in the *CLN3* mRNA, Whi3-S568A would not be able to bind to the *CLN3*^{mGCAU} mRNA, and so Whi3-S568A cells harboring *CLN3*^{mGCAU} 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 Δ*cln3* Whi3-HA cells harboring the *CLN3*^{mGCAU} mRNA allele was smaller than that of those harboring *CLN3* (Fig. 6*B*). As expected, the size of Δ*cln3* Whi3-S568A-HA cells harboring the *CLN3*^{mGCAU} mRNA allele was also similarly smaller than that of the control *CLN3*, as in the case of the Δ*cln3* Whi3-HA cells (Fig. 6*B*). In contrast, the *CLN3*^{mGCAU} mRNA had no significant effect on the size of the Δ*cln3* Whi3-S568D-HA or Δ*cln3* Δ*whi3* cells (Fig. 6*B*). 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 G_1/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 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 Δ*whi3* mutation, the S568D mutation accelerated

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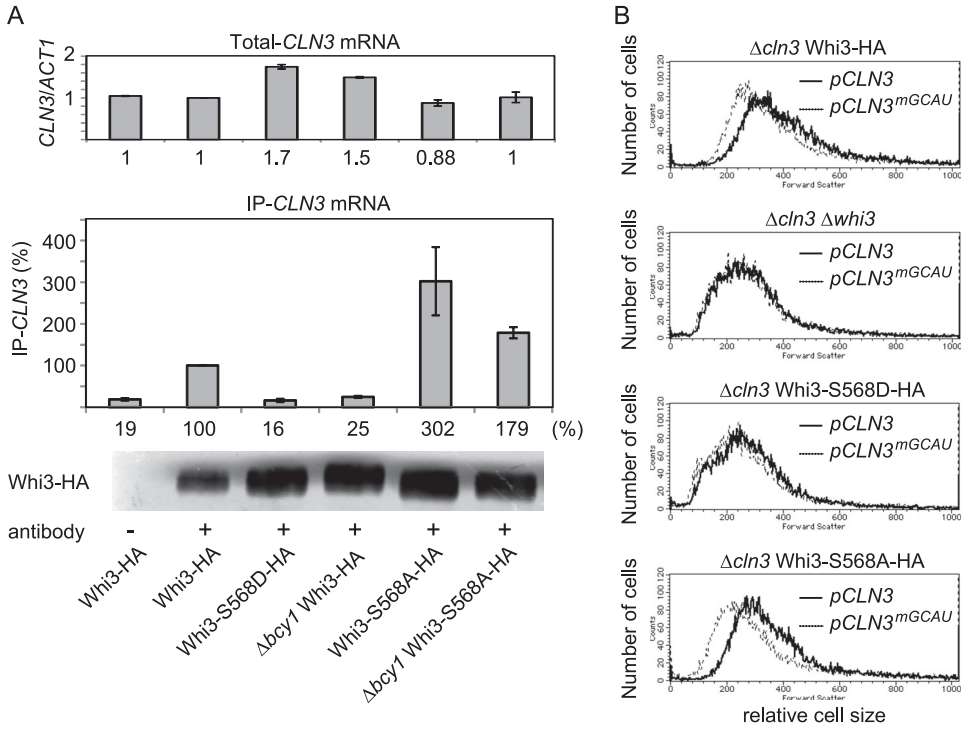

FIGURE 6. **Phosphorylation of Whi3 by PKA inhibits binding of Whi3 to *CLN3* mRNA *in vivo*.** *A*, extracts of cells expressing Whi3-HA, Whi3-S568D-HA, Whi3-S568A-HA, Δ*bcy1* Whi3-HA, or Δ*bcy1* Whi3-S568A-HA were immunoprecipitated as described under "Experimental Procedures." Each sample was separated 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-PCR. The relative intensity of the *CLN3* mRNA was normalized to that of *ACT1* mRNA (*Total*). Values relative to the *CLN3* mRNA level of Whi3-HA (+) are indicated below each bar. Data represent the means ± S.E. of three independent experiments. *B*, Δ*cln3* Whi3-HA, Δ*cln3* Δ*whi3*, Δ*cln3* Whi3-S568D-HA, or Δ*cln3* Whi3-S568A-HA cells expressing a wild-type (p*CLN3*) or mutant (p*CLN3*^{mGCAU}) construct on centromeric vectors were grown in synthetic complete medium (−Ura + 2% dextrose) to early log phase, and cell size of samples was determined using a FACSCalibur.

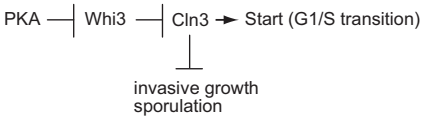

FIGURE 7. **Model for the role of PKA in regulating multiple cellular functions by phosphorylating Whi3 at Ser-568.**

the G_1/S transition (Fig. 4). In addition, we demonstrated that the S568D mutation was defective in invasive growth and sporulation as clearly as the Δ*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 G_1 in response to glucose (14). Conversely, a sudden change from a poor carbon source to glucose leads to a G_1 delay by activation of PKA to maintain a critical cell size (12, 13). This discrepancy may lie in the different effects on G_1 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 G_1 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*B*), 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 Δ*bcy1* strain showed reduced interaction with *CLN3* mRNA (Fig. 6*A*).

We have shown that the *WHI3-S568D* allele behaved like the *whi3* null mutation in all *in vivo* assays described here. However, prominent 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.

Role of Whi3 via PKA in Multiple Cellular Events

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*[S]

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In eukaryotic cells, 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)[2] 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 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, *scz14*, 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 (*MAT*a *trp1 leu2 ade2 ura3 his3 can1–100*), YAT1 (*MAT*a *zds1*Δ::*TRP1*), YJY10 (*MAT*a *zds1*Δ::*TRP1 sir3*Δ::*kanMX4*), YJY9 (*MAT*a *sir3*Δ::*kanMX4*), YGA13 (*MAT*a *pmc1*Δ::*kanMX4*), YGA14 (*MAT*a *pmr1*Δ::*kanMX4*), DHT14 (*MAT*a *cnb1*Δ::*HIS3*), YJY12 (*MAT*a *cnb1*Δ::*HIS3 sir3*Δ::*kanMX4*), DMY2798 (*MAT*a *leu2*::*mURA3-LEU2*), YSS3 (*zds1*Δ::*KanMX4* in DMY2798), YGA4 (*cnb1*Δ::*KanMX4* in DMY2798), YSS8 (*sir3*Δ::*KanMX4* in DMY2798), YGA9 (*cnb1*Δ::*HIS3 sir3*Δ::*KanMX4* in DMY2798), DMY2827 (*sir2*Δ::*KanMX4* in DMY2798), DMY2800 (*MAT*a *NTS2*::*mURA3-LEU2*), YSS5 (*zds1*Δ::*KanMX4* in DMY2800), YGA6 (*cnb1*Δ::*KanMX4* in DMY2800), YSS10 (*sir3*Δ::*KanMX4* in DMY2800), YGA11 (*cnb1*Δ::*HIS3 sir3*Δ::*KanMX4* in DMY2800), DMY2831 (*sir2*Δ::*KanMX4* in DMY2800), DMY2804 (*MAT*a *NTS1*::*mURA3-LEU2*), YSS4 (*zds1*Δ::*KanMX4* in DMY2804), YGA5 (*cnb1*Δ::*KanMX4* in DMY2804), YSS9 (*sir3*Δ::*KanMX4* in DMY2804), YGA10 (*cnb1*Δ::*HIS3 sir3*Δ::*KanMX4* in DMY2804), DMY2835 (*sir2*Δ::*KanMX4* in DMY2804), DMY2895 (*MAT*a *adh4*::*URA3*), YSS1 (*zds1*Δ::*KanMX4* in DMY2895), YGA2 (*cnb1*Δ::*KanMX4* in DMY2895), YSS6 (*sir3*Δ::*KanMX4* in DMY2895), YGA7 (*cnb1*Δ::*HIS3 sir3*Δ::*KanMX4* in DMY2895), DMY2841 (*sir2*Δ::*KanMX4* in DMY2895), DMY2896 (*MAT*a *TEL VIIL*::*URA3*), YSS2 (*zds1*Δ::*KanMX4* in DMY2896), YGA3 (*cnb1*Δ::*KanMX4* in DMY2896), YSS7 (*sir3*Δ::*KanMX4* in DMY2896), YGA8 (*cnb1*Δ::*HIS3 sir3*Δ::*KanMX4* in DMY2896), and DMY2839 (*sir2*Δ::*KanMX4* in DMY2896) were the derivatives of strain W303-1A. Also used were YG880 (*hmr*Δ*E::TRP1 TEL VR::URA3 RDN1::ADE2-CAN1 his3 leu2*), YG882A (*zds1*Δ::*KanMX4* in YG880), YG884A (*cnb1*Δ::*KanMX4* in YG880), YG883A (*sir3*Δ::*KanMX4* in YG880), YG885A (*cnb1*Δ::*HIS3 sir3*Δ::*KanMX4* in YG880), and YG881 (*sir2*Δ::*KanMX4* in YG880).

Gene Disruption and Strain Construction—The *zds1*Δ, *cnb1*Δ, *sir2*Δ, and s*ir3*Δ strains were constructed by gene replacement. Genomic DNA was isolated from the *zds1::kanMX4, cnb1::kanMX4, sir2::kanMX4,* and *sir3::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 on 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.

Silencing Assays—Silencing assays were performed as described previously (33, 34). Cells were grown in nonselective liquid medium, and serial dilutions were spotted onto plates. The plates were incubated for 2–3 days at 25 °C and then photographed.

RESULTS

*Deleting SIR3 Confers Ca^{2+} Resistance to zds1*Δ *Cells*—The growth of *ZDS1* null mutant (*zds1*Δ) yeast cells in medium containing 300 mM $CaCl_2$ is severely inhibited, with the cells exhibiting G_2 arrest and highly polarized bud growth. As shown in supplemental Fig. 1, the growth inhibition and polarized bud growth but not the G_2 delay of *zds1*Δ cells because of exogenous $CaCl_2$ were suppressed by an additional *scz14* mutation. The plasmid that complemented the phenotype of the *scz14* mutants was cloned, and the mutant gene was identified. Genetic linkage analysis showed that the *scz14* mutation resided in the *SIR3* gene (data not shown). The deletion of the *SIR3* gene, similar to the *scz14* mutation, suppressed the growth inhibition and the polarized bud growth of *zds1*Δ cells, suggesting that the *scz14* mutation is a loss of function allele of the *SIR3* gene (Fig. 1, *A–C*). Therefore, we used the *sir3* deletion (*sir3*Δ) in further experiments.

Why did the *sir3*Δ mutation suppress only the Ca^{2+}-induced hyperpolarized bud growth but not the G_2-delay? We showed previously that the Ca^{2+} signal induces polarized bud growth by elevating the level of G_1-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 *sir3*Δ (compare *zds1*Δ and *zds1*Δ *sir3*Δ in Fig. 1*D*). The suppression of the Ca^{2+} sensitivity of the *zds1*Δ 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 *sir3*Δ disruption on the Cln2 level was seen on the WT background (compare WT and *sir3*Δ in Fig. 1*D*). These results indicate that the *sir3*Δ 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. 2*A*). 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

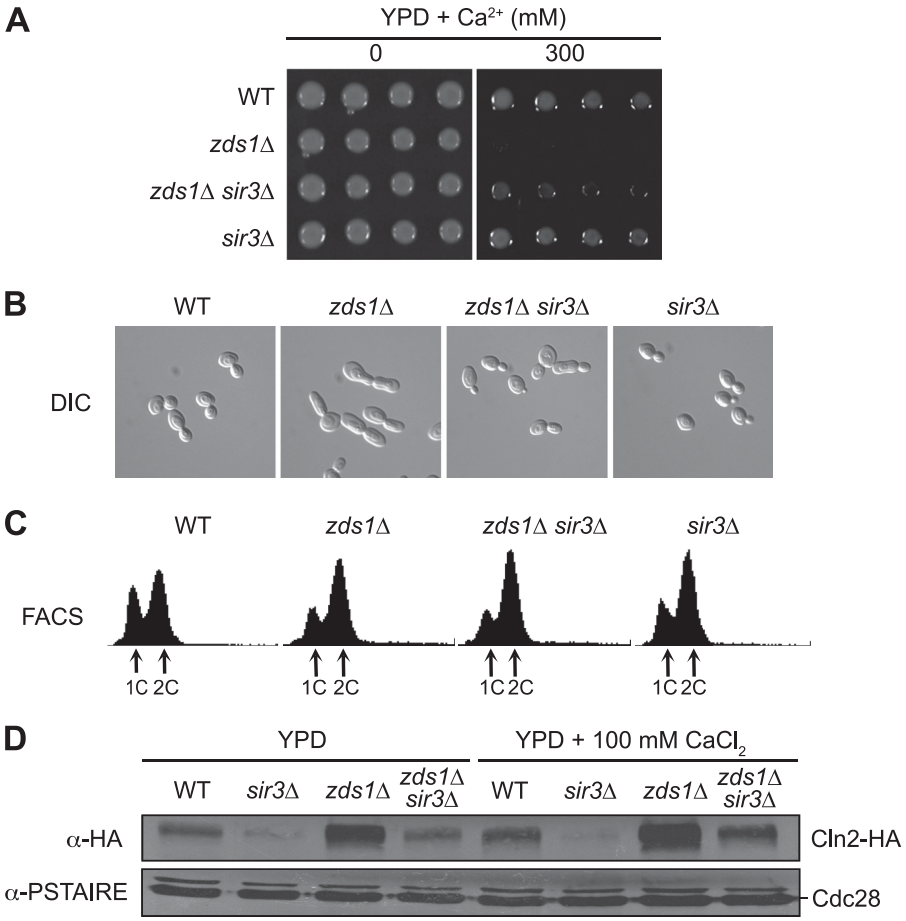

FIGURE 1. **The deletion of the *SIR3* gene suppresses various phenotypes of the *zds1*Δ strain.** *A*, effect of the deletion of the *SIR3* gene on the growth of the *zds1*Δ mutant strain on solid medium. WT, *sir3*Δ, *zds1*Δ, and *zds1*Δ *sir3*Δ cells were spotted on YPD solid medium containing 300 mM $CaCl_2$, after which the plates were incubated at 25 °C for 2 days. *B* and *C*, cell morphology after 6 h of incubation with 100 mM $CaCl_2$ at 25 °C (*B*). Flow cytometry analysis (FACS) of propidium iodide-stained cells of various strains after 6 h of incubation with 100 mM $CaCl_2$ at 25 °C (*C*). *DIC*, differential interference contrast; *1C*, one DNA copy; *2C*, two DNA copies. *D*, Cln2-HA and Cdc28 were detected by Western blotting using early log phase growing cells of those strains indicated in *A*, suspended in YPD containing 100 mM $CaCl_2$, incubated for 6 h, and then used for Western blotting. Cln2-HA or Cdc28 was detected by immunoblotting with anti-HA or anti-PSTAIRE antibody, respectively.

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. 2*A*). 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*Δ 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*Δ cells was shorter than that of WT cells under the experimental conditions (Fig. 2*B*), although it was reported previously that the mean RLS of *zds1*Δ cells was longer than that of WT cells (24).

To investigate the activation of Ca^{2+} signaling pathways in *zds1*Δ cells under the experimental conditions, we estimated the calcineurin activity *in vivo* by using a reporter assay system (31). In response to the elevation of the cellular Ca^{2+} level, the transcription factor Crz1 binds to the calcineurin-dependent response element in the promoter and activates several stress-responsive target genes. We used the calcineurin-dependent response element-driven *LacZ* reporter gene (31) and measured the calcineurin activity in the WT and *zds1*Δ cells under normal growth condition (YPD) by β-galactosidase assay. The β-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*Δ) (Fig. 2*C*). As expected, β-galactosidase activity in the *zds1*Δ cells increased significantly by 3-fold compared with that in WT cells (Fig. 2*C*), and this increase was indeed dependent on calcineurin (Fig. 2*C*, compare *zds1*Δ and *zds1*Δ *cnb1*Δ). These results indicate that the increase in cellular Ca^{2+} level in the *zds1*Δ 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 $CaCl_2$ sensitivity of the *zds1*Δ 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.

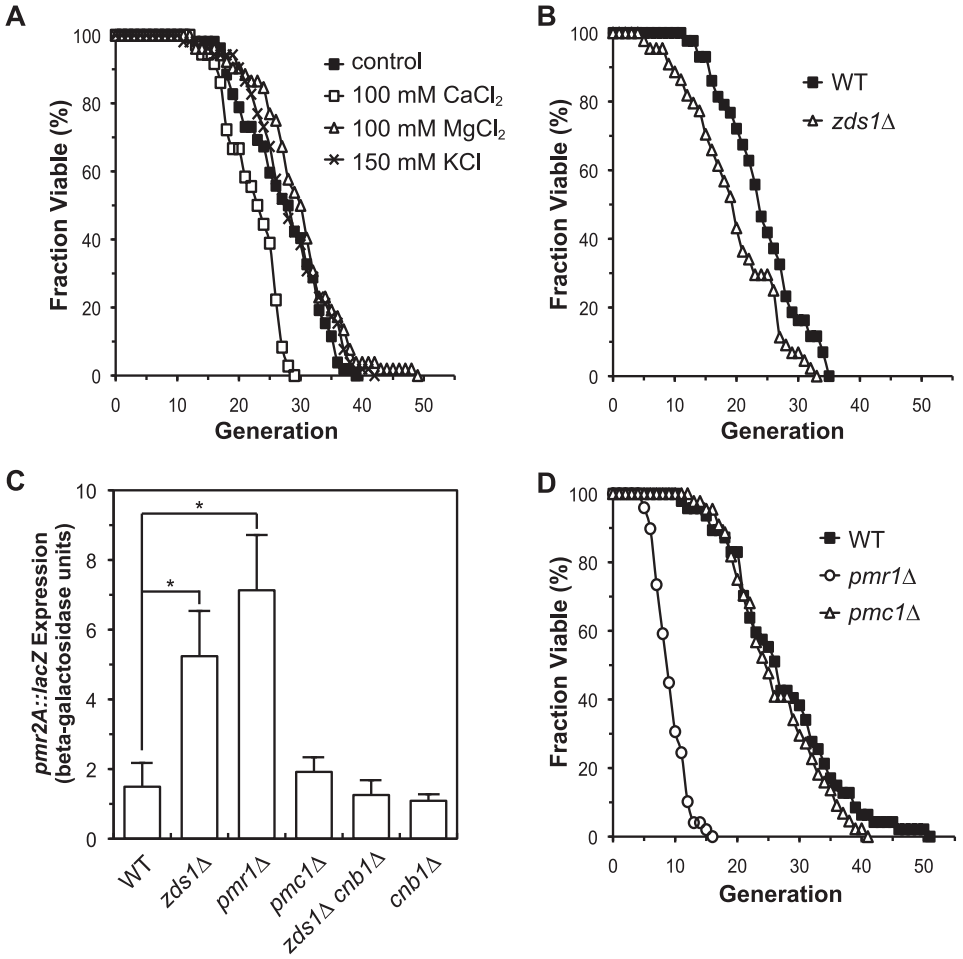

FIGURE 2. **An increase in Ca^{2+} levels led to a reduced RLS.** *A*, RLS analysis of WT cells grown on YPD (*control*), YPD plus 100 mM $CaCl_2$, YPD plus 100 mM $MgCl_2$, and YPD plus 150 mM KCl at 25 °C. The mean RLS in terms of numbers of cell divisions are control, 27.4 ($n = 52$); 100 mM $CaCl_2$, 22.5 ($n = 36$); 100 mM $MgCl_2$, 29.8 ($n = 52$); and 150 mM KCl, 28.3 ($n = 52$). *B*, RLS analysis of WT and *zds1*Δ mutant cells grown on YPD at 25 °C. The mean RLSs are WT, 24.3 ($n = 43$) and *zds1*Δ, 19.6 ($n = 44$). *C*, expression of the calcineurin-dependent response element-dependent reporter gene in WT, *zds1*Δ, *zds1*Δ *cnb1*Δ, *pmc1*Δ, *pmr1*Δ, and *cnb1*Δ mutant cells. *, $p < 0.001$. *D*, RLS analysis of WT, *pmc1*Δ, and *pmr1*Δ mutant cells grown on YPD at 25 °C. The mean RLSs are WT, 27.5 ($n = 47$); *pmc1*Δ, 26.3 ($n = 44$); and *pmr1*Δ, 9.4 ($n = 49$).

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

Hyperactivation of Calcineurin Reduces the RLS—To further confirm the above suggestion that activation of Ca^{2+} 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. 3*A*). This result established that activation of calcineurin signaling shortens the RLS.

*Calcineurin Deletion Overcomes the Shortened RLS of zds1*Δ *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 Ca^{2+} (25). As shown in Fig. 2*B*, 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 *zds1*Δ cells. As expected, the mean RLS of *zds1*Δ *cnb1*Δ double deletion cells was longer than that of *zds1*Δ single deletion cells (Fig. 3*B*). Interestingly, the mean RLS of *zds1*Δ *cnb1*Δ 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 *zds1*Δ 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

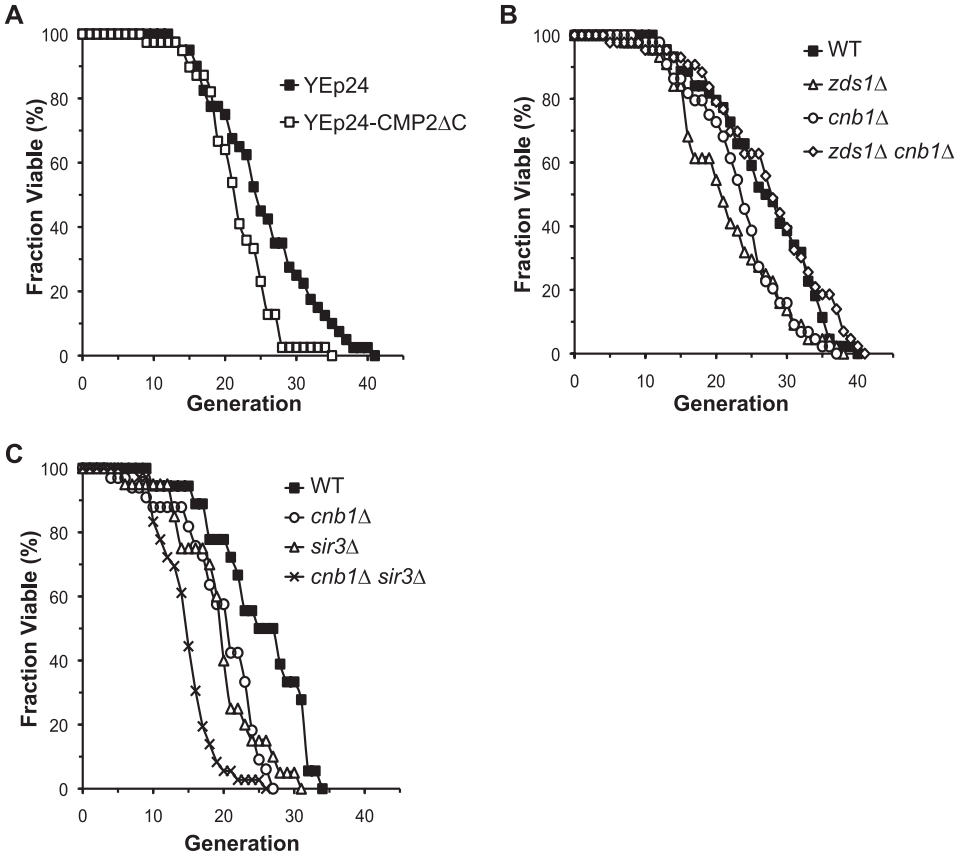

FIGURE 3. **Effect of overexpression and deletion of calcineurin on the RLS.** *A*, RLS analysis of the cells overexpressing the constitutively active form of calcineurin. WT cells transformed with empty plasmid (*YEp24*) or YEp24-CMP2ΔC plasmid grown on YPD at 25 °C were examined. Mean RLSs are YEp24, 25.5 ($n = 40$) and YEp24-CMP2ΔC, 21.9 ($n = 39$). *B*, RLS analysis of WT, *zds1*Δ, *cnb1*Δ, and *zds1*Δ *cnb1*Δ mutant cells grown on YPD at 25 °C. The mean RLSs are WT, 26.9 ($n = 44$); *zds1*Δ, 21.8 ($n = 44$); *cnb1*Δ, 23.5 ($n = 44$); and *zds1*Δ *cnb1*Δ, 27.6 ($n = 43$). *C*, RLS analysis of WT, *cnb1*Δ, *sir3*Δ, and *cnb1*Δ *sir3*Δ mutant cells grown on YPD at 25 °C. The mean RLSs are WT, 25.2 ($n = 18$); *cnb1*Δ, 19.7 ($n = 33$); *sir3*Δ, 19.6 ($n = 20$); and *cnb1*Δ *sir3*Δ, 15.0 ($n = 36$).

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 *cnb1*Δ *sir3*Δ. As reported previously (20), deletion of *SIR3* resulted in a 22% decrease in the mean RLS (Fig. 3*C*). The mean RLS of *cnb1*Δ *sir3*Δ double deletion cells was shortened more compared with that of each single deletion mutant (Fig. 3*C*). Thus, it appears that calcineurin and Sir3 act redundantly in the regulation of the RLS as parallel pathways.

Calcineurin Does Not Affect Silencing at the rDNA, Telomeres, or Mating-type Locus—To further seek the calcineurin-related regulation of RLS, we examined whether calcineurin is involved in gene silencing. Silencing at *NTS1* has been proposed to counteract rDNA recombination, a phenomenon that has been implicated in yeast aging (33). In budding yeast, each 9.1-kb repeat yields a 35 S precursor rRNA (transcribed by RNA polymerase (Pol) I) and a 5 S rRNA (transcribed by RNA Pol III), separated by two nontranscribed spacers, *NTS1* and *NTS2* (52). To investigate whether calcineurin affects silencing at the rDNA, we constructed the *CNB1*-deleted cells carrying an *mURA3* reporter gene integrated into one of the following sites: outside of the rDNA array at the non-silencing *LEU2* locus (euchromatic locus) or within the rDNA unit at the strong silencing region (*NTS1* or *NTS2* locus) (34). As reported (33, 34), 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 inserted at the euchromatic locus (Fig. 4*A*). As reported previously, defective silencing was observed in the *sir2*-deleted cells (Fig. 4*A*). No change was observed in the growth of *cnb1*Δ cells on -URA medium plates as in WT cells (Fig. 4*A*), indicating that calcineurin did not affect the silencing at a rDNA region.

Next, to investigate whether calcineurin was required for silencing at other heterochromatic regions, we examined silencing at telomeres and the mating-type locus in the *cnb1*-deleted cells. We constructed the *CNB1*-deleted cells, in which a *URA3* reporter gene was integrated into the telomeric repeats of Chromosome VIIL or the non-silencing *ADH4* locus as the 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 *URA3*-expressing cells (53). Both the WT and the *cnb1*Δ cells were able to grow on the 5-fluoroorotic acid medium, whereas the *sir2*Δ and *sir3*Δ strain, in which silencing fails and *URA3* is expressed, did not grow at all (Fig. 4*B*). Further, to monitor silencing of the mating-type locus, we used a strain with a *TRP1* marker integrated into the *HMR* locus. Similar to the effect at other regions

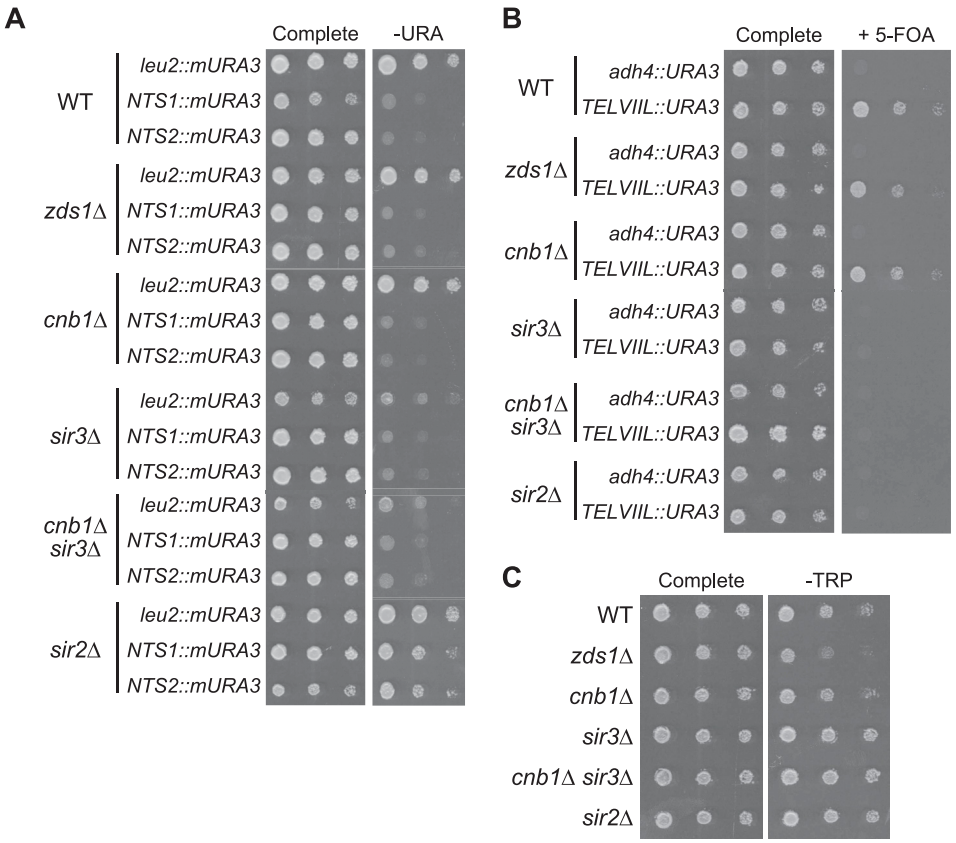

FIGURE 4. **Effect of the deletion of calcineurin (*cnb1*Δ) on silencing at rDNA, telomeres, and the mating-type locus.** *A*, silencing at rDNA. 5-fold serial dilutions of cultures of the indicated strains were spotted onto -URA medium. The synthetic complete medium (*Complete*) was using strains containing an *mURA3* insertion at outside rDNA, *NTS1*, or *NTS2*. *SIR2* is required for silencing at *NTS1* and *NTS2*. *B*, silencing at telomeres. 5-fold serial dilutions of cultures of the indicated strains were spotted onto synthetic complete medium containing 5-fluoroorotic acid (+*5-FOA*) to assess telomeric silencing. *C*, silencing at mating-type locus. 5-fold serial dilutions of cultures of the indicated strains were spotted onto synthetic complete medium lacking tryptophan (-*TRP*).

(rDNA and telomeres), the silencing at the mating-type locus was normal in the Δ*cnb1* cells (Fig. 4*C*). 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. Hyperactivation of calcineurin signaling reduced the RLS. Indeed, the shortened RLS of *zds1*Δ 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 *zds1*Δ strain were abolished by the calcineurin deletion mutations (25). In addition, calcineurin activity was enhanced in the *zds1*Δ cells (Fig. 2*D*). 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 *zds1*Δ deletion reduced the RLS. However, previously, the another group reported that the *zds1*Δ 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 *zds1*Δ cells would be caused by the differences in the experimental conditions. Further, it was reported that the *zds1*Δ strain on the BY4742 background had no effect on the RLS (54). We noted that the *zds1*Δ cells on the W303 background were more sensitive to Ca^{2+} than those on the BY4742 background.[3] It is possible that the short-lived *zds1*Δ cells on W303 were due to the elevated level of intracellular Ca^{2+}.

Why did the *pmr1*Δ cells exhibit a severe reduction in mean RLS compared with other cells (*zds1*Δ, *cnb1*Δ, and *sir3*Δ)? The

[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 secretary pathway has not been identified yet in the systematic genetic screening for the RLS regulator, it has been reported that some proton ATPase mutants, such as the *VMA* genes encoding vacuolar H_1-ATPases, reduced the RLS (57). These results suggest that the severe RLS reduction of *pmr1*Δ 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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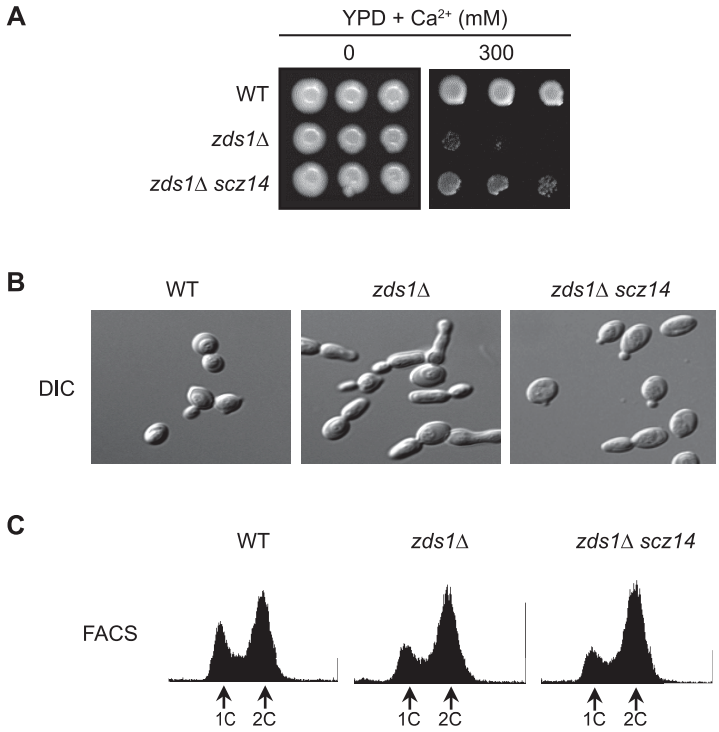

<u>Supplementary Fig. 1.</u> The *scz14* mutation, a mutant allele of *SIR3* gene, suppresses various phenotypes of the *zds1*Δ strain. (A) Effect of *scz14*, mutant alleles of *SIR3*, on the growth of the *zds1*Δ mutant strain on solid medium. WT, *scz14*, and *zds1*Δ *scz14* cells were spotted on YPD solid medium containing 300 mM $CaCl_2$, 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 $CaCl_2$ at 25ºC (B). Flow cytometry analysis (FACS) of PI-stained cells of various strains after 6 h of incubation with 100 mM $CaCl_2$ at 25ºC (1C: 1 DNA copy or 2C: 2 DNA copies) (C).

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(1) Evidence for antagonistic regulation of restart from G_1 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 G_1 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 G_1, but the mechanism by which cells restart from G_1 delay remains elusive. We found that Whi3, a negative regulator of G_1 cyclin, counteracted Hog1 in the restart from G_1 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 Δ*whi3* strain, slightly suppressed G_1 delay of Δ*hog1* cells under osmotic stress conditions, whereas the non-phosphorylatable S568A mutation of Whi3 caused prolonged G_1 arrest of Δ*hog1* cells. These results indicate that Hog1 activity is required for restart from G_1 arrest under osmotic stress conditions, whereas Whi3 acts as a negative regulator for this restart mechanism.

Key words: G_1 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 G_1 cell-cycle arrest by a dual mechanism that involves downregulation of G_1 cyclin transcription and direct phosphorylation of CDK-inhibitor Sic1,[5] but the mechanism by which the cells restart from G_1 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 G_1/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 adenylyl 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 G_1 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

Table 1. Strains Used in This Study

Strain	Genotype	Source or reference
W303 strain		
W303-1A	*MAT***a** *ade2-1 his3-11, 15 leu2-3, 112, trp1-1, ura3-1 can1-100*	Rothstein
SOS5	*MAT***a** Δ*hog1::TRP1*	16
SHI338	*MAT***a** Δ*hog1::TRP1 sgh4*	17
SHI336	*MAT***a** *sgh4*	17
SHI301	*MAT***a** Δ*whi3::kanMX6*	This study
SHI177	*MAT***a** Δ*cln3::URA3*	This study
SHI305	*MAT***a** Δ*hog1::TRP1* Δ*whi3::kanMX6*	This study
SHI402	*MAT***a** Δ*hog1::TRP1* Δ*whi3::kanMX6* Δ*cln3::URA3*	This study
SHI403	*MAT***a** Δ*hog1::TRP1* Δ*whi3::kanMX6* Δ*cln1::kanMX6* Δ*cln2::LEU2*	This study
SHI179	*MAT***a** Δ*hog1::TRP1* Δ*cln3::URA3*	This study
SHI411	*MAT***a** Δ*hog1::TRP1 WHI3-3HA::kanMX6*	This study
YMM503	*MAT***a** Δ*hog1::TRP1 WHI3 S568A-3HA::kanMX6*	This study
YMM506	*MAT***a** Δ*hog1::TRP1 WHI3 S568D-3HA::kanMX6*	This study
YMM522	*MAT***a** Δ*cln3::URA3* Δ*whi3::kanMX6*	This study

RT-PCR Kit II (Takara, Kyoto, Japan). Total RNA was isolated by use of an RNeasy 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 $CaCl_2$ due to hyper-activation of Ca^{2+}-signaling and high osmolarity.[16,17] To identify the genes involved in this regulatory mechanism, we screened for mutants that suppress the growth defect of a *hog1*-deleted (Δ*hog1*) strain on solid medium containing a high concentration of $CaCl_2$. Recessive, Ca^{2+}-resistant mutants were classified into six loci, designated *sgh1-sgh6* (for suppressors of Ca^{2+}-induced growth defect of *hog1*). Although these mutations were originally isolated based on their ability to suppress the $CaCl_2$ sensitivity of Δ*hog1* cells, four mutations (*sgh3*/*tup1*, *sgh5*/*sko1*, *sgh6*/*cyc8*, and *sgh4*), but not two others (*sgh1*/*swe1* 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.[17] In this present study, we characterized mutation *sgh4*.

By crossing *sgh4* Δ*hog1* strain with the parental strain, the *sgh4* strain was constructed. The cell sizes of the *sgh4* strain and of the *sgh4* Δ*hog1* 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 Δ*hog1*-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 (Δ*whi3*) 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 G_1 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 G_1 cyclins *CLN1* and *CLN2*, which leads to a delay in the start of the cell cycle.[7,8,10,18] 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 Δ*hog1* cells by the *whi3* deletion was caused by upregulation of Cln3. As expected, the suppression of the osmotic sensitivity of Δ*hog1* 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 G_1-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 Δ*hog1* strain (Fig. 1C). Moreover, the Δ*cln3* and Δ*whi3* Δ*cln3* 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 G_1 cyclin, whereas Whi3 negatively regulated G_1 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 Δ*hog1* cells in normal YPD medium were similar in size, but under osmotic conditions (a medium containing 0.4 M KCl), the Δ*hog1* cells were larger than the WT cells (Fig. 1D). This increase in size of the Δ*hog1* cells was reversed by the *whi3* deletion (Fig. 1D, Δ*hog1* Δ*whi3*), further, an additional *cln3* deletion in the Δ*hog1* Δ*whi3* double mutant again caused an increase in cell size (Fig. 1D). These results indicate that the increase in cell size of Δ*hog1* under osmotic conditions was caused by Whi3-

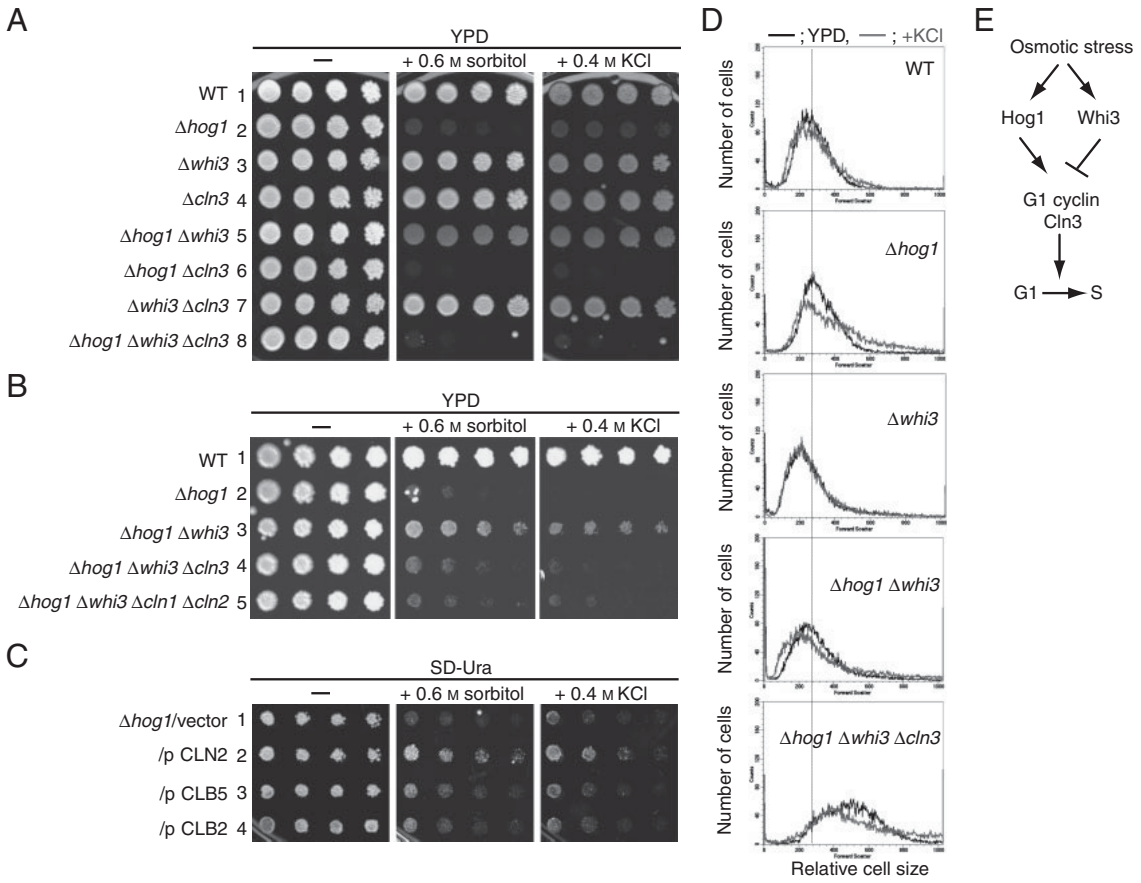

Fig. 1. Inhibition of Whi3 Was Required for Cell Growth under Osmotic Sress to Promote the Action of G_1 Cyclins.

A, Growth of various strains on solid YPD medium containing 0.6 M sorbitol or 0.4 M KCl. Serial dilutions of wild-type (WT), Δ*hog1*, Δ*whi3*, Δ*cln3*, Δ*hog1* Δ*whi3*, Δ*hog1* Δ*cln3*, Δ*whi3* Δ*cln3*, and Δ*hog1* Δ*whi3* Δ*cln3* cells were spotted onto plates, which were then incubated for 3 d at 28 °C. B, Growth of various strains on solid YPD medium containing 0.6 M sorbitol or 0.4 M KCl. Serial dilutions of WT, Δ*hog1*, Δ*hog1* Δ*whi3*, Δ*hog1* Δ*whi3* Δ*cln3*, and Δ*hog1* Δ*whi3* Δ*cln1* Δ*cln2* cells were spotted onto plates, which were then incubated for 3 d at 28 °C. C, Growth of various strains on solid SD-Ura medium containing 0.6 M sorbitol or 0.4 M KCl. Serial dilutions of the Δ*hog1* strain transformed with a multi-copy plasmid (YEplac195) harboring each of the cyclin genes (empty vector, *CLN2*, *CLB5*, and *CLB2*) were spotted onto plates, which were then incubated for 3 d at 28 °C. D, Cell size under the osmotic-stress conditions. WT, Δ*hog1*, Δ*whi3*, Δ*hog1* Δ*whi3*, Δ*hog1* Δ*cln3*, and Δ*hog1* Δ*whi3* Δ*cln3* cells were grown in YPD medium (black line) or in one with 0.4 M KCl (gray line) for 6 h at 28 °C. Relative cell size was determined by measuring forward angle light scattering by FACSCalibur (Becton Dickinson, Tokyo, Japan). Histograms were obtained by examining 2×10^4 cells in each case. Pathway by which Hog1 and Whi3 might affect G_1 cyclin function in response to osmotic stress. E, Antagonistic regulation of G_1 cyclin in response to osmotic stress by the Hog1 and Whi3. The arrows and the blunt-ended line indicate positive and negative controls respectively.

dependent downregulation of G_1 cyclin. Thus the regulation of G_1 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 G_1 delay

To clarify the role of the regulatory pathway of G_1 cyclin *via* Whi3 in cell-cycle control under osmotic conditions, we examined carefully the cell-cycle progression of cells released from the synchronized G_1 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 Δ*hog1* cells exhibited delays in G_1/S transition (Fig. 2A and B), bud formation, and the onset of nuclear division (Fig. 2C). As previously reported,[3] osmotic stress-induced G_1 delay in the Δ*hog1* cells was longer than that in the WT cells, suggesting that Hog1 is required for restart from G_1 delay (Fig. 2B). Further, deletion of the *WHI3* gene partially suppressed G_1 delay in the Δ*hog1* strain (Fig. 2B). This suggests that when Whi3 is inhibited, cells can restart from osmotic stress-induced G_1 delay. Moreover, when we used a higher concentration of KCl (0.6 M), the G_1/S transition of Δ*whi3* 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 G_1 cell-cycle control under osmotic stress conditions.

The phosphomimetic Whi3-S568D mutation slightly suppressed the defect in restart from the osmotic stress-induced G_1 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.[10] It has also been reported that PKA is upregulated at the transcriptional level in response to environmental stresses such as heat shock.[19] 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

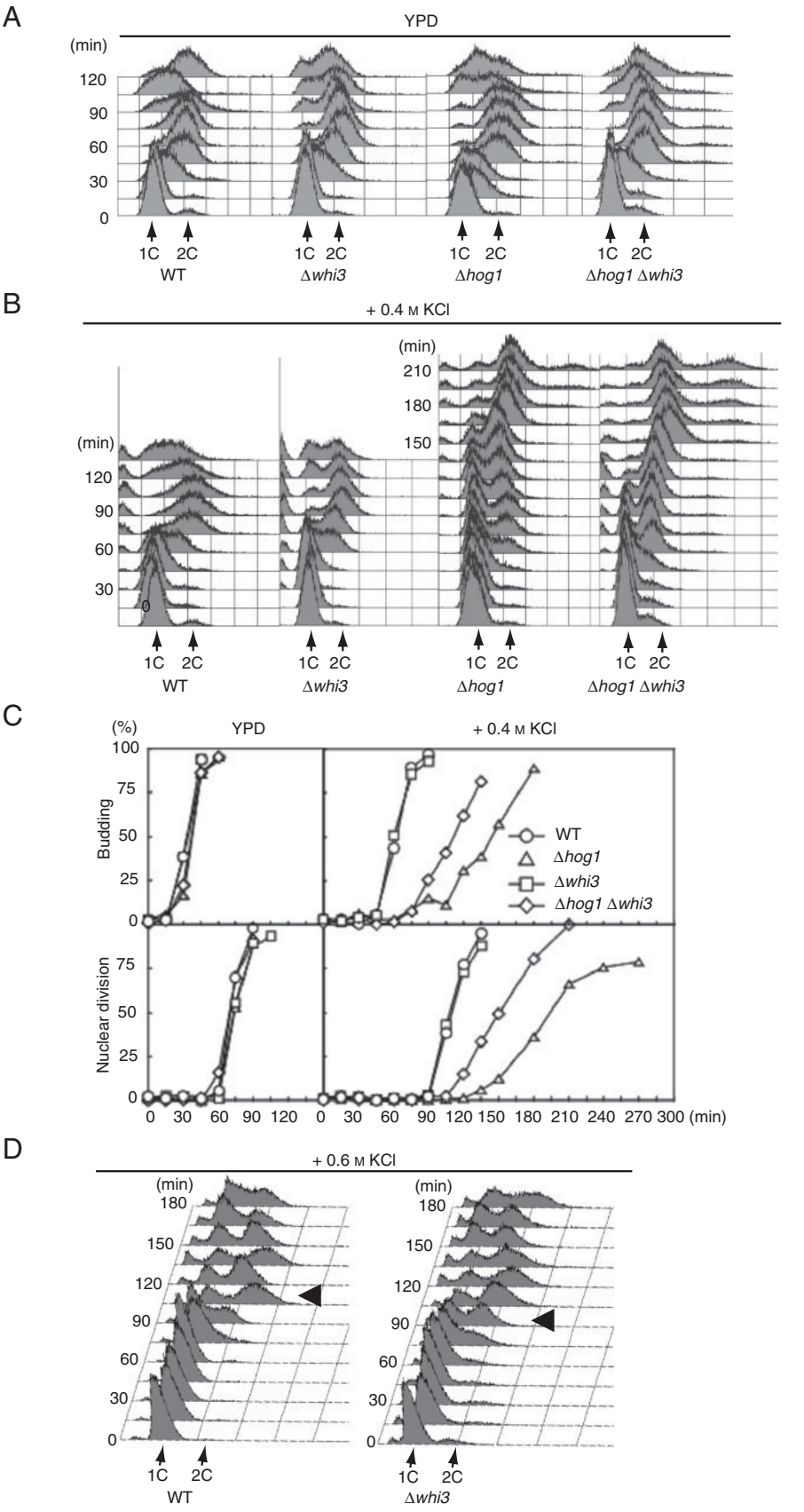

Fig. 2. Inhibition of Whi3 Was Required for Restart after Osmotic Stress-Induced G_1 Delay.

A and B, Cellular DNA levels of the WT, Δ*whi3*, Δ*hog1*, and Δ*hog1* Δ*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, Δ*hog1*, Δ*whi3*, and Δ*hog1* Δ*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 Δ*whi3* strains synchronized with α-factor and released into YPD medium with 0.6 M KCl. Solid triangles indicate the period of G_1/S transition for each strain.

well established as a transcriptional regulator in the osmotic stress response.[1)] To investigate further whether the transcriptional activation of *TPK1* in response to osmotic stress was dependent on Hog1, we performed the same experiment using Δ*hog1* cells. The results indicated that the *TPK1* mRNA level in the Δ*hog1* 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.

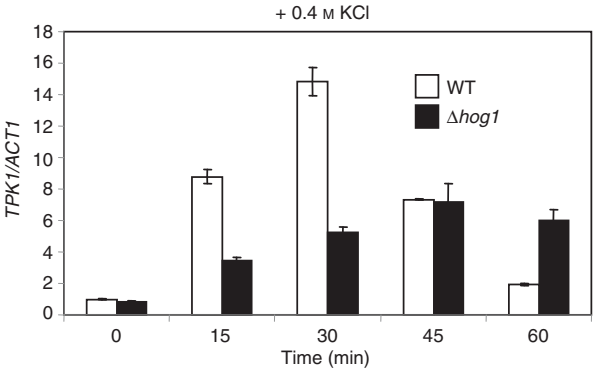

Fig. 3. *TPK1* mRNA Was Transiently Upregulated by Osmotic Stress in a Hog1-Dependent Manner.

TPK1 and *ACT1* (control) mRNA levels in WT and Δ*hog1* 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.

Because previous work has shown that phospho-mimic Whi3-S568D mutant cells behave like Δ*whi3* cells,[10)] we speculated that the Whi3-S568D mutation would suppress the G_1 delay of Δ*hog1* 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 Δ*hog1* cells under osmotic stress conditions (0.2 and 0.6 M KCl). As reported previously,[10)] under normal growth conditions, the Whi3-S568D single mutation led to a shortened G_1 phase as in the case of Δ*whi3* cells, whereas the Whi3-S568A single mutation led to a prolonged G_1 phase (data not shown). As expected, the Whi3-S568D mutation slightly suppressed the G_1 delay of Δ*hog1* cells in a medium containing 0.2 M KCl (Fig. 4A, arrowheads, compare to Δ*hog1* Whi3 and Δ*hog1*Whi3-S568D for 45–60 min). We noted that the suppressive effect of the Whi3-S568D mutation on the

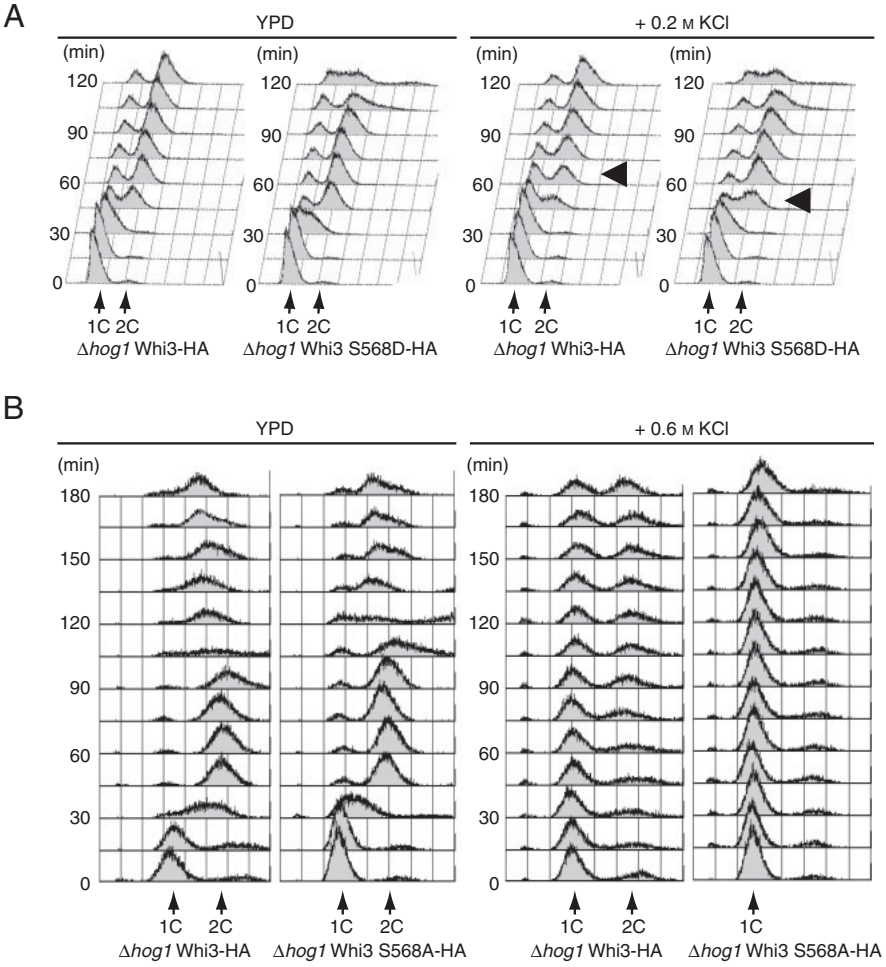

Fig. 4. The Phosphomimetic Whi3-S568D Mutation Slightly Suppressed the G_1 Delay in *hog1* Mutant upon Exposure to High Osmolarity.

A, Effects of Whi3 S568D mutation on cell-cycle progression. The Δ*hog1* Whi3-HA and Δ*hog1* Whi3-S568D-HA strains were synchronized with α-factor, released into YPD medium with and without 0.2 M KCl, and incubated at 28 °C for up to 120 min. Solid triangles indicate the period of G_1/S transition for each strain. B, Effects of the Whi3-S568A mutation on cell-cycle progression. The Δ*hog1* Whi3-HA and Δ*hog1* Whi3-S568A-HA strains were synchronized with α-factor, released into YPD with and without 0.6 M KCl, and incubated at 28 °C for up to 180 min.

cell-cycle progression of the Δ*hog1* cells was much weaker than that of *whi3* deletion (compare Figs. 2B and 4A). Further, the Whi3-S568A mutation caused prolonged G_1 arrest of Δ*hog1* cells in a medium containing 0.6 M KCl (Fig. 4B), as for Whi3 wild-type cells under osmotic stress conditions (Fig. 2B, Δ*hog1* cells). Together, these observations suggest that inhibition of Whi3 is required for restart from osmotic stress-induced G_1 delay.

Discussion

We suggest that Hog1 SAPK pathway and the Whi3 cell-cycle regulator antagonize restart from G_1 delay following osmotic stress. Upon exposure of Δ*hog1* cells to osmotic stress, the cells exhibited a severe G_1 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 G_1 delay was caused by a low level of G_1 cyclins through activation of Whi3, a negative regulator of G_1 cyclin. Thus our results suggest that Hog1 activity is important for restart/recovery from G_1 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 G_1 delay caused by osmotic stress (Fig. 1E). Transcription of *TPK*, a budding yeast PKA Tpk1 that directly phosphorylates and inactivates Whi3,[10] was upregulated under osmotic stress in a Hog1-dependent manner (Fig. 3). Further, the phospho-mimicing Whi3 S568D mutant slightly suppressed the G_1 delay of Δ*hog1* cells under high-osmolarity conditions (Fig. 4A). Thus, it is possible that Hog1 is involved in the restart from osmotic stress-induced G_1 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 G_1 arrest in response to moderately hyperosmotic stress.[3] It has been reported that Hog1 activation is required for G_1 arrest in response to hyperosmotic stress.[5] Here, we found that a G_1 delay was induced by osmotic stress even in Δ*hog1* cells (Fig. 2B, Δ*hog1*), indicating that the cells have a Hog1-independent mechanism inducing G_1 delay in response to osmotic stress. Hog1 regulates a number of molecules in cell-cycle control (both G_1 arrest and restart from G_1) under osmotic stress conditions. A previous study indicated that expression of a constitutively active form of Hog1 is capable of inducing G_1 arrest under normal growth conditions,[5] suggesting that a Hog1-induced mechanism for G_1 arrest might be dominant to that for restart from G_1.

The partial rescue of the sensitivity of Δ*hog1* 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 G_1 delay in Δ*hog1* 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.

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バイオミディア

パン酵母でアンチエイジング

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老化は誰にでも起きる現象であり，どんなに偉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だろうか？その夢を実現する妙薬はまだ発見されていないが，少なくとも老化を遅らせることはできるようだ．というのも，複雑な老化の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を明らかにするうえで，酵母，線虫，マウスといったいわゆる真核モデル生物を用いた研究が必要不可欠である．実際，さまざまなモデル生物を用いた研究によって，その謎のひとつひと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く，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，私たちにも馴染み深い生物である．しかしながら，そもそも出芽酵母が老化するのかという疑問をもつ人も多いかもしれない．出芽酵母には，「複製的寿命」[1]と「経時的寿命」[2]という2つの寿命がある．1つの細胞が一生の間に分裂できる回数には限りがあり，最終的には娘細胞を産むことができなくなる（複製的寿命）．また，分裂しない細胞でも，その生存率は日を追うごとに減少していく（経時的寿命）．これらが出芽酵母の老化であり，老化に伴っていろいろな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年老いて，最終的には死を迎える．

出芽酵母の老化は，細胞にと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．環状のリボソームDNA（ERC）や，酸化または凝集してしま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．出芽酵母のリボソーム遺伝子は100以上の繰り返し配列で構成されたrDNA領域といわれる部分に存在する．ここで起きたコピーミス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Sir2を余分にもたせると寿命を延ばすことができ，一方その形成を促すFob1を欠損させることにより寿命を延ばせることが報告されている[3,4]．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．驚いたことに，実は細胞の中身も非対称的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[5]．これによって，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，老化のリセットが観察されており，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．

老化を遅延させる手だては他にもある．そのひとつがカロリー制限である．アンチエイジングに興味がある人なら，一度は聞いたことがあるかもしれない．これは摂取カロリーを低減することで寿命を延ばす方法で，酵母をはじめとした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，培地に含むグルコースを2%から0.5%に減らすと約30%の寿命延長効果が得られる．カロリー摂取を減らすことで寿命が延び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わっ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，生育に必要な細胞外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，上述したシグナル伝達経路が欠損した変異株は寿命が延長することが知られているが，これらの変異株にカロリー制限を行っても，寿命がさらに延びることはない．また，カロリー制限の際，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がカロリー制限の効果と同じ効果をもっていることを裏付ける[6]．

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ごく一部を紹介した．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大きな注目が集まっている．いつもは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に長生きできる日も近いだろう．

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