Systematic analysis of Ca\(^{2+}\) homeostasis in \textit{Saccharomyces cerevisiae} based on chemical-genetic interaction profiles

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**ABSTRACT** We investigated the global landscape of Ca\(^{2+}\) homeostasis in budding yeast based on high-dimensional chemical-genetic interaction profiles. The morphological responses of 62 Ca\(^{2+}\)-sensitive (cls) mutants were quantitatively analyzed with the image processing program CalMorph after exposure to a high concentration of Ca\(^{2+}\). After a generalized linear model was applied, an analysis of covariance model was used to detect significant Ca\(^{2+}\)-cls interactions. We found that high-dimensional, morphological Ca\(^{2+}\)-cls interactions were mixed with positive (86%) and negative (14%) chemical-genetic interactions, whereas one-dimensional fitness Ca\(^{2+}\)-cls interactions were all negative in principle. Clustering analysis with the interaction profiles revealed nine distinct gene groups, six of which were functionally associated. In addition, characterization of Ca\(^{2+}\)-cls interactions revealed that morphology-based negative interactions are unique signatures of sensitized cellular processes and pathways. Principal component analysis was used to discriminate between suppression and enhancement of the Ca\(^{2+}\)-sensitive phenotypes triggered by inactivation of calcineurin, a Ca\(^{2+}\)-dependent phosphatase. Finally, similarity of the interaction profiles was used to reveal a connected network among the Ca\(^{2+}\) homeostasis units acting in different cellular compartments. Our analyses of high-dimensional chemical-genetic interaction profiles provide novel insights into the intracellular network of yeast Ca\(^{2+}\) homeostasis.

**INTRODUCTION**
Calcium is an essential component of eukaryotic cells. Although present in low concentration, intracellular Ca\(^{2+}\) is important for structural, enzymatic, and signaling roles in many cellular pathways (Williams, 1999). High levels of cytosolic Ca\(^{2+}\) are considered damaging because of precipitation with organic anions. For this reason, eukaryotic cells have a variety of homeostatic mechanisms to maintain cellular homeostasis.

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effectively acquire, use, and store Ca\textsuperscript{2+} and keep intracellular levels of Ca\textsuperscript{2+} at a nontoxic level (Cyert and Philipott, 2013).

The budding yeast Saccharomyces cerevisiae has been used as a eukaryotic model organism to study Ca\textsuperscript{2+} homeostasis. In S. cerevisiae, the concentration of free cytosolic Ca\textsuperscript{2+} is maintained between 50 and 200 nM, whereas its concentration in the environment ranges from <1 µM to >100 mM (Halachmi and Eilam, 1993; Cui and Kaandorp, 2006; Cui et al., 2009). A key element in Ca\textsuperscript{2+} homeostasis is its intracellular compartmentalization. Ca\textsuperscript{2+} pools are classified as exchangeable or nonexchangeable based on pulse-chase experiments (Eilam, 1981). Most of the nonexchangeable pools reside in cellular compartments, such as the vacuole and the endoplasmic reticulum (ER), and the exchangeable pools are believed to be located in the cytosol (Eilam, 1981; Eilam et al., 1985; Tanida et al., 1996).

The vacuole is the primary Ca\textsuperscript{2+}-storage organelle in yeast, containing >90% of the intracellular Ca\textsuperscript{2+} (Dunn et al., 1994). A vacuolar Ca\textsuperscript{2+}-translocating ATPase, Pmc1p, plays an important role in the sequestration of cytosolic Ca\textsuperscript{2+} into the vacuole, andPMC1 mutants exhibit reduced vacuolar Ca\textsuperscript{2+} content, resulting in a Ca\textsuperscript{2+}-sensitivity growth phenotype (Cunningham and Fink, 1994). This PMC1 mutant phenotype is aggravated by disruption of the vacuolar H\textsuperscript{+}-Ca\textsuperscript{2+} exchanger Vcx1p because Pmc1p and Vcx1p coordinate to maintain cytosolic Ca\textsuperscript{2+} homeostasis. Calmodulin, a major intracellular Ca\textsuperscript{2+}-binding protein, is ubiquitous in eukaryotic cells, and the Ca\textsuperscript{2+}/calmodulin signaling pathway responds to elevated cytosolic Ca\textsuperscript{2+}. In response to the increased signal, Ca\textsuperscript{2+}/calmodulin binds to calcineurin, the Ca\textsuperscript{2+}/calmodulin-dependent phosphatase. Calcineurin up-regulates transcription of PMC1 via activation of the transcription factor Crz1p and negatively regulates Vcx1p by an unknown mechanism (Cunningham and Fink, 1996; Stathopoulos and Cyert, 1997; Pittman et al., 2004). Vacuolar proton-translocating ATPase (V-ATPase) VMA genes are also important for Ca\textsuperscript{2+} homeostasis (Ohya et al., 1991; Umemoto et al., 1991; Bachhawat et al., 1993; Hirata et al., 1993; Ho et al., 1993). Studies have shown that the proton-motive force driven by V-ATPase is required for activation of Ca\textsuperscript{2+} transporters in the vacuole, including Vcx1p (Cunningham and Fink, 1996; Pozos et al., 1996; Förster and Kane, 2000). The vacuole also has a countering Ca\textsuperscript{2+} release channel, Yvc1p, which depends on elevated cytosolic Ca\textsuperscript{2+} levels for its activity (Chang et al., 2010). Overexpression of Yvc1p results in elevation of cytosolic Ca\textsuperscript{2+} and Ca\textsuperscript{2+} sensitivity (Denis and Cyert, 2002).

In addition to the vacuole, the ER and the plasma membrane are also involved in Ca\textsuperscript{2+} homeostasis. The Ca\textsuperscript{2+} concentration in the lumen of the ER is much higher than in the cytosol (Demaurex and Frieden, 2003) and the ER contains several Ca\textsuperscript{2+}-dependent enzymes. Calcium-sensitive 2p (Cls2p)/Caq2p encodes a regulatory subunit of mannosylinositol phosphophorylceramide (MIPC) synthase, which is involved in sphingolipid metabolism and the regulation of Ca\textsuperscript{2+} homeostasis in the ER lumen (Beeler et al., 1994; Takita et al., 1995; Tanida et al., 1996). The plasma membrane has an additional mechanism for the regulation of Ca\textsuperscript{2+} influx called a high-affinity Ca\textsuperscript{2+} influx system (HACS), which is composed of at least three components, including Cch1p, Ecm7p, and Mid1p (Cunningham, 2011). Maintaining Ca\textsuperscript{2+} homeostasis through the HACS is necessary for adaptation and survival of the cells during mating processes (Iida et al., 1994; Fischer et al., 1997; Martin et al., 2011).

A genetic interaction refers to a phenotype caused by combining the effects of individual genetic variants. In general, based on the differences between observed and expected double-mutant phenotypes, genetic interactions can be classified into two groups, those with positive or negative interactions (Dixon et al., 2009). A negative genetic interaction describes a double mutant in which the phenotype is stronger than expected. The most extreme case of a negative fitness interaction is synthetic lethality, in which the combination of two mutations results in an inviable phenotype (Mani et al., 2008; Baryshnikova et al., 2013). Similar to genetic interactions, a chemical-genetic interaction refers to a phenotype caused by combining the effects of chemicals and genetic variants. Chemical synthetic lethality is a negative interaction in which a chemical compound causes lethality in a particular mutant. On the other hand, a positive genetic interaction indicates a double mutant with less defective fitness than expected (Mani et al., 2008; Baryshnikova et al., 2013). Along the same line, a positive chemical-genetic interaction is a suppression or masking of effects of a chemical compound in a particular mutant.

Genetic analysis of Ca\textsuperscript{2+}-sensitive (cls) mutants in yeast uncovered a variety of genes required for Ca\textsuperscript{2+} homeostasis and Ca\textsuperscript{2+} tolerance. Genome-wide screening of the cls mutants unable to grow in the presence of 100 mM CaCl\textsubscript{2} (Ohya et al., 1986b; Sambade et al., 2005; Ohnuki et al., 2007; Zhao et al., 2013) has led to the comprehensive identification of components directly or indirectly involved in intracellular Ca\textsuperscript{2+} homeostasis. More than 60 cls mutants defective in Ca\textsuperscript{2+} homeostasis have been isolated, such as pmc1, vma, and cls2 mutants (Ohya et al., 1986b; Ohnuki et al., 2007). Note that cls mutants exhibit various growth phenotypes, including divalent cation and pH sensitivity and carbon source utilization. pmc1 mutants exhibit a Ca\textsuperscript{2+}-specific sensitive phenotype, whereas vma mutants exhibit broad sensitivity to Ca\textsuperscript{2+}, Mn\textsuperscript{2+}, and Zn\textsuperscript{2+}. Our and other studies have indicated that cls mutants exhibit various unique morphological phenotypes in Ca\textsuperscript{2+}-rich conditions (Yu et al., 1996; Ohnuki et al., 2007; Yoshida et al., 2013). These results suggested that theCLS genes have diverse cellular functions, including cell morphogenesis, and the entire gene network, involved in Ca\textsuperscript{2+} homeostasis, is not yet fully understood.

The present study investigates the global landscape of Ca\textsuperscript{2+} homeostasis in yeast by analyzing the high-dimensional Ca\textsuperscript{2+}–cls interaction profiles of 62 cls yeast mutants. To identify Ca\textsuperscript{2+}–cls interaction profiles, we quantified the cellular, nuclear, and actin morphology of the 62 cls mutants with the image processing system CalMorph (Ohtani et al., 2004; Ohya et al., 2005) in the presence and absence of 100 mM CaCl\textsubscript{2}. The obtained high-dimensional morphological data were used to define negative and positive interactions between Ca\textsuperscript{2+} and Ca\textsuperscript{2+}-sensitive mutations. In addition, extensive statistical analyses of the interaction profiles revealed functional groups of CLS genes, functional correlations between the groups, and a global view of Ca\textsuperscript{2+} homeostasis and the Ca\textsuperscript{2+} regulatory network.

RESULTS
Identification of Ca\textsuperscript{2+}–cls interactions using high-dimensional morphological phenotypes
Because high concentrations of Ca\textsuperscript{2+} induce morphological changes in the cls mutants, we used morphological characteristics to detect interactions between Ca\textsuperscript{2+} and the cls mutations (Ohnuki et al., 2007). Ca\textsuperscript{2+}-induced morphological changes in the cls mutants are different from the Ca\textsuperscript{2+}–cls interactions, which are a combinatorial effect of Ca\textsuperscript{2+} and the cls mutation defined as the differences in the Ca\textsuperscript{2+}-induced morphological changes between wild type and the cls mutant, which has been defined in previous genetic interaction studies (Dixon et al., 2009; Jonkas et al., 2009). For each of the 501 morphological parameters, the most appropriate probability distribution model was selected from the Gaussian, gamma, beta, and
beta-binominal as previously described (Yang et al., 2014), and a generalized linear model was used to standardize the high-dimen-
sional morphological data of wild-type cells and the 62 cls mutants. Analysis of covariance (ANCOVA) revealed significant Ca\(^{2+}\)-cls interactions in 209 morphological parameters (p < 0.05, likelihood ratio test between two linear models with/without the Ca\(^{2+}\)-cls interaction term), which were summarized into 19 independent morpholog-
ical features (\(\pi\) score), and used for further analysis (\(\pi > 0\) for a positive inter-
action; \(\pi < 0\) for a negative interaction).

Feature analysis of the Ca\(^{2+}\)-cls interactions

To understand further the physiological implica-
tion of Ca\(^{2+}\)-cls interactions, we per-
formed a feature analysis. First, the majority of the cls mutants exhibited more positive interactions than negative interactions (Supple-
mental Figure S2A). We found that 88.7% (55 of 62) of cls mutants exhibited more positive interactions than negative. In other words, 85.73% (1382 of 1612 significant interactions out of 7006 assays, 62 cls mutants \(\times\) 113 parameters, at \(p < 0.05\) by Wald test, and false discovery rate [FDR] = 0.24) of the detected interac-
tions were positive. It is notable that all fitness-related Ca\(^{2+}\)-cls interactions were negative because Ca\(^{2+}\) has aggravating effects on the growth of Ca\(^{2+}\)-sensitive mutants. Second, the number of Ca\(^{2+}\)-cls interactions was not correlated with that of Ca\(^{2+}\)-induced morpho-
logical changes in the cls mutants (Ohnuki et al., 2007; Supplemental Figure S2B). The number of Ca\(^{2+}\)-cls interactions was less corre-
lated (\(R = 0.27, p < 0.05\), Student’s t test) with that of significantly changed parameters in each mutant (Mann–Whitney U test, \(p < 0.01\), implying that Ca\(^{2+}\)-cls interactions had different implications than with Ca\(^{2+}\)-induced morphological changes (for complementary information, see Ohnuki et al., 2007). Third, only a small proportion of cls mutants exhibited many Ca\(^{2+}\)-cls interactions. Although the number of Ca\(^{2+}\)-cls interactions varied, only 3.22% of the interactions changed bidirectionally.
Ca\(^{2+}\). Thus, the Ca\(^{2+}\)--cls interaction profiles likely provide a unique source for the understanding of various Ca\(^{2+}\) responses in the cls mutants.

**Clustering analysis of high-dimensional Ca\(^{2+}\)--cls interaction profiles**

To test whether similarities of the Ca\(^{2+}\)--cls interaction profiles can be used for functional classification of the CLS genes, we first extracted the interaction profiles of 16 PCs from 209 parameters by principal component analysis (PCA) and performed clustering analysis with the multiscale bootstrap technique (Suzuki and Shimodaira, 2006; Figure 3). The analysis showed that nine classes, with a total of 49 cls mutants (79%), were statistically identified (the approximately unbiased probability value [AU p] estimates the probability of observation of a given class in infinite number of classes >0.95; for more information, see Shimodaira, 2002; Figure 3). The defective cellular functions in the mutants were several classes were annotated by gene ontology (Supplemental Table S1). For example, class I consists of 17 V-ATPase–defective mutants (Ohya et al., 1991; Kane, 2006). Class II involves several mutants in the core subunits of homotypic fusion and protein sorting (HOPS) and class C core vacuole/endo-some tethering (CORVET) complexes involved in membrane fusion at endosomes, vacuoles, and lysosomes (Balderhaar and Ungermann, 2013). Class IV contains the mutants cls4-1 and bem1, which are defective in Ca\(^{2+}\)--modulated bud formation (Yoshida et al., 2013). Class VIII includes fei3 and frt1, which are defective in iron transport (Kwok et al., 2006). We also noted that the cls mutants with related Ca\(^{2+}\)--cls interaction profiles have similar intracellular Ca\(^{2+}\) content. The intracellular Ca\(^{2+}\) content in class I, with the exception of vma22, was 0.6-fold less than that of wild-type cells, whereas the intracellular Ca\(^{2+}\) content of the cls II mutants was 3.1-fold greater than wild type (Supplemental Table S1). Moreover, some cls mutants with similar Ca\(^{2+}\)--cls interaction profiles showed similar divergent cation sensitivity; 29 mutants showed Ca\(^{2+}\)-specific sensitivity, such as mutants of IV, VIII, and IX classes, whereas all of the vma mutants showed broad divergent cation sensitivity (Supplemental Tables S1 and S2). These results suggested that the majority of the cls mutants with related Ca\(^{2+}\)--cls interaction profiles have defects in a similar function.

**Riboflavin deficiency of class VI cls mutants results in failure to maintain Ca\(^{2+}\) homeostasis**

To examine further whether cls mutants with related Ca\(^{2+}\)--cls interaction profiles have defects in a similar function, we focused on the class VI cls mutants. Class VI contains gla1, rib4, and yel045c, all of which showed a slow growth phenotype in yeast extract/polypeptone/dextrose (YPD) medium and a severe growth defect in Ca\(^{2+}\)-rich medium. RIB4 and GLY1 encode lumazine synthase and threonine aldolase, respectively, which play a role in the riboflavin biosynthetic pathway. Lumazine synthase synthesizes a precursor of riboflavin (Monschau et al., 1998), and overproduction of threonine aldolase increases riboflavin content (Garcia-Ramirez et al., 1995). YEL045c is a dubious open reading frame in the promoter region of GLY1. Because the levels of riboflavin are likely reduced in all mutants, we tested whether the Ca\(^{2+}\)-sensitive phenotype of the class VI cls mutants was suppressed by riboflavin. We found that the Ca\(^{2+}\)-sensitive growth phenotype of rib4 was completely suppressed by riboflavin, whereas the other class VI mutants were only partially suppressed (Figure 4A), suggesting that riboflavin is required for Ca\(^{2+}\) homeostasis.

To identify whether the class VI cls mutants are defective in a particular pathway, we examined vacuolar acidification and sphingolipid metabolism in these mutants. In contrast with the V-ATPase mutants, the class VI mutants have intact quinacrine-positive vacuoles (Figure 4B), suggesting that riboflavin is not critical for vacuolar acidification. We examined the ability to synthesize sphingolipids after wild-type and mutant cells were cultured in YPD medium containing \(^{3}H\) palmitic acid for 90 min. The lipids were extracted and separated by thin-layer chromatography (TLC). Consistent with a previous report (Uemura et al., 2003), deletion of the CSG2 gene, which encodes MIPC synthase, resulted in a drastic decrease in the amount of MIPC and M(IP)\(_2\)C and an increase in the amount of IPC (Figure 4C). On the other hand, the lipid composition of the class VI cls mutants was quite similar to that of wild-type cells, suggesting that riboflavin is not important for MIPC synthase activity (Figure 4C). We then examined whether failure of Ca\(^{2+}\) homeostasis results in an energy imbalance, by measuring the amount of NADP\(^+\) and NADPH. We found that class VI cls mutants have elevated ratios of NADP\(^+\)/NADPH concentrations (Figure 4D). The ratio of the concentrations of NADP\(^+\)/NADPH of cls2/csg2 was similar to that of wild-type cells. Thus, class VI mutants all exhibited a riboflavin deficiency, causing an elevation of NADP\(^+\), possibly due to a failure to maintain Ca\(^{2+}\) homeostasis.
Characterization of Ca\textsuperscript{2+}–cls interactions in each functional gene unit

To understand further each class of mutants, we compared positive and negative Ca\textsuperscript{2+}–cls interactions in each class (Figure 5A). The 1612 significant interactions (out of 7006 assays; 62 cls mutants × 113 parameters) were identified at $p < 0.05$ by Wald test, where the permutation test showed that $<387$ interactions were expected to be detected by chance (FDR = 0.24). The total number of Ca\textsuperscript{2+}–cls interactions was biased, depending on the class. In particular, cls mutants of classes I (V-ATPase), II (class C vacuolar protein sorting or C-VPS), and IV (Ca\textsuperscript{2+}-modulated bud formation) showed 9.22, 8.62, and 5.77 times more average interaction density, respectively, than class VIII (Fe ion transporter; Figure 5A, inset). Therefore, the genes of classes I, II, and IV cls mutations may be important under conditions of high Ca\textsuperscript{2+}. Note that class I and II cls mutations resulted in severe growth defects in Ca\textsuperscript{2+}-rich medium (Supplemental Table S1). Negative interactions were more frequently observed in particular classes: class III and IV cls mutants showed a high proportion of negative Ca\textsuperscript{2+}–cls interactions (Figure 5B), on average 8.8 and 7 times higher, respectively, than class I cls mutants (Figure 5B, inset). This suggested that each class of mutants has a unique pattern of negative and positive Ca\textsuperscript{2+}–cls interactions.

Class IV cls mutants shared three significant negative Ca\textsuperscript{2+}–cls morphological parameter interactions, which can be attributed to S/G2 and M phases of the cell cycle (Supplemental Figure S3A). Class III cls mutants also had three significant negative interactions attributed to G1 and S/G2 phases (Supplemental Figure S3B). Considering that class IV mutations sensitize morphological responses in S/G2 and M phases of the cell cycle and class III mutations sensitize morphological responses in G1 and S/G2 phases, it seemed that sensitized responses during the cell cycle are class specific. This suggested that the morphology-based negative interactions are unique signatures that represent sensitized cellular processes and pathways.

Responses of the Ca\textsuperscript{2+}-sensitive mutants to calcineurin inhibition

Calcineurin is a Ca\textsuperscript{2+}/calmodulin-dependent phosphatase important for several cellular functions, including Ca\textsuperscript{2+} homeostasis (Cyert, 2003). To understand each class of mutants with respect to calcineurin regulation, we systematically investigated the Ca\textsuperscript{2+}-sensitive phenotypes of the cls mutants after inactivation of calcineurin. For this purpose, we used the immunosuppressant drug FK506 (Heitman et al., 1991), which specifically inhibits calcineurin activity. Previous studies showed that inactivation of calcineurin confers either alleviating or aggravating effects on cell growth: FK506 suppressed the Ca\textsuperscript{2+}-sensitive growth of pmc1 (Cunningham and Fink, 1994), whereas it enhanced that of vma mutants (Tanida et al., 1995). We found that the Ca\textsuperscript{2+}-sensitive phenotype of 13 cls mutants (21%) was suppressed, whereas that of 40 cls mutants (65%) was enhanced (Figure 6A, Supplemental Figures S4 and S5, and Supplemental Table S2). Of interest, the cls mutants with FK506-enhanced phenotypes accumulated either high or low intracellular Ca\textsuperscript{2+} content (Supplemental Figure S6). Thus, the majority of the cls mutants with severely altered Ca\textsuperscript{2+} homeostasis showed FK506-enhanced phenotypes, consistent with previous reports (Tanida et al., 1995; Cunningham and Fink, 1996). Each mutant class had a common response to FK506. Class I, II, and III cls mutants showed FK506-enhanced Ca\textsuperscript{2+} sensitivity, whereas classes IV, VI, and VIII mutants showed FK506-suppressed Ca\textsuperscript{2+}-sensitivity (Supplemental Table S1). This suggested that inactivation of calcineurin has similar effects on cls mutants with similar functional defects, which supports functional classification of the CLS genes based on similarities of the Ca\textsuperscript{2+}–cls interaction profile as well.
FK506-suppressed and -enhanced cls mutants discriminated well after the interaction profile PCA. We found that cls mutants were distinguishable in two-dimensional Ca\(^{2+}\)-cls interaction space, particularly in the first and second PCs (PC1 and PC2, respectively; Figure 6B and Supplemental Figure S7). FK506-enhanced class I and II mutants are all plotted in the right side with higher PC1 scores and class III with lower PC2 scores. Representative morphological features of PC1 and PC2 are shown in Supplemental Figure S8. Thus, Ca\(^{2+}\)-cls interaction profiles can be used to discriminate between suppression and enhancement of the Ca\(^{2+}\)-sensitive phenotypes triggered by inactivation of calcineurin.

**DISCUSSION**

We applied high-dimensional chemical-genetic interaction profiles to gain a systems-level understanding of Ca\(^{2+}\) homeostasis in *S. cerevisiae*. Ca\(^{2+}\)-induced morphological changes in Ca\(^{2+}\)-sensitive mutants were quantified from 501 points of view and analyzed to obtain interaction profiles between Ca\(^{2+}\) and Ca\(^{2+}\)-sensitive mutations. Interaction profiles were clustered into nine groups linked to cellular processes involved in vacuolar acidification, cell morphogenesis, Ca\(^{2+}\)-modulated bud formation, riboflavin biogenesis, protein sorting, and Fe ion transportation, as well as unknown processes...
in the cytoplasm, nucleus, and ER. On the basis of the analysis of the Ca\(^{2+}\)-cls interaction profiles, we constructed a systems-level view of Ca\(^{2+}\) homeostasis. Because the deletion mutants of all the nonessential genes in the yeast genome were screened for Ca\(^{2+}\)-sensitive mutants, this study provides a blueprint for a global understanding of the factors required for Ca\(^{2+}\) homeostasis because it covers all the genes essential for proliferation in conditions of high Ca\(^{2+}\).

**Positive and negative Ca\(^{2+}\)-cls interactions based on morphological changes**

In analogy with genetic interactions, combinatorial effects of chemical components and genetic variants to generate a phenotype are known as chemical-genetic interactions. In addition, a negative interaction (chemical synthetic lethality) defines as lethal effects of a chemical compound in a given mutant. Ca\(^{2+}\)-sensitive mutants showed deleterious effects on growth in conditions of high Ca\(^{2+}\), exhibiting a negative interaction with Ca\(^{2+}\).

In this study, high-dimensional morphological data were employed to describe negative and positive interactions between Ca\(^{2+}\) and Ca\(^{2+}\)-sensitive mutants. Whereas fitness Ca\(^{2+}\)-cls interactions were all negative, morphological Ca\(^{2+}\)-cls interactions were positive and negative, although there were more positive interactions. A negative interaction is interpreted as a Ca\(^{2+}\) response sensitized by the cls mutation, whereas a positive interaction is caused by suppression, masking changes of the cls mutation. Thus, Ca\(^{2+}\)-cls interaction profiles based on morphological changes will likely provide unique data sources.

Studies of several model organisms have proposed that many genes have a few genetic interactions, whereas a small number of genes are highly connected to one another and serve as network hubs (Dixon et al., 2009). Consistent with this, this study revealed that a small number of CLS genes exhibit a large number of Ca\(^{2+}\)-cls interactions. The number of Ca\(^{2+}\)-cls interactions was strongly biased in the nine functional classes. In particular, classes I (V-ATPase), II (C-VPS), and IV (Ca\(^{2+}\)-modulated bud formation) exhibited a large number of Ca\(^{2+}\)-cls interactions. Note that the same classes (I, II, and IV) of cls mutations resulted in severe growth defects in Ca\(^{2+}\)-rich medium. Therefore, the CLS genes with many Ca\(^{2+}\)-cls interactions have a large effect, serving as hubs that play key roles in cell proliferation and morphogenesis in conditions of high Ca\(^{2+}\).

**CLS genes with similar Ca\(^{2+}\)-cls interaction patterns share intracellular functions**

Genetic interaction profiles have been used to classify genes based on intracellular functions, proposing that genes that belong to a given biological pathway or process tend to share similar genetic interaction profiles (Tong et al., 2004; Schuldiner et al., 2005; Pan et al., 2006; Jonikas et al., 2009; Costanzo et al., 2010, 2016). Using hierarchical clustering based on the Ca\(^{2+}\)-cls interaction scores, we identified nine functional gene units in Ca\(^{2+}\) homeostasis and Ca\(^{2+}\)-regulatory pathways. This indicated that the CLS genes with the same cellular function showed a similar pattern of morphological Ca\(^{2+}\)-cls interactions.

Clustering analysis of 59 CLS genes was performed previously based on the similarity of Ca\(^{2+}\)-induced morphological changes (Ohnuki et al., 2007). Fifty-one percent of the cls mutants were classified into seven groups, and three groups were functionally
The identified clusters can be used to predict the function of the uncharacterized CLS genes. One of the examples is class VI, to which three genes, Rif4, Gly1, and YEL045C, were assigned. Rif4 is required for riboflavin biosynthesis, which is a precursor of flavin adenine dinucleotide (FAD; Oltmanns and Bacher, 1972). Gly1p is the glycine biosynthetic enzyme threonine aldolase (McNeil et al., 1994), and YEL045C is a dubious open reading frame in the promoter region of the GLY1 gene. In the filamentous fungus Ashbya gossypii, which is closely related to yeast, production of riboflavin was improved by overexpression of the GLY1 gene (Monschau et al., 1998), suggesting that Gly1p is involved in riboflavin biosynthesis. The Ca\(^{2+}\)-sensitive growth phenotype of the class VI cls mutants was suppressed by the addition of riboflavin. Although the molecular mechanism remains to be elucidated, all class VI cls mutants exhibited defects not in vacuolar acidification or sphingolipid biosynthesis, but in perturbation of the energy balance of NADPH.

The class II cls mutants, where cls5-1 was classified with vps11, vps16, vps18, and vps33, are known to be core subunits of the CORVET and HOPS complexes, which function in endosome–endosome fusion and homotypic vacuole fusion (Balderhaar and Ungermann, 2013). Although CLSS (PFY1) encodes profilin, which is required for actin organization, both intracellular Ca\(^{2+}\) content and initial Ca\(^{2+}\) uptake were elevated in cls5-1 mutants and four other Ca\(^{2+}\)-sensitive mutants (Ohya et al., 1986b), suggesting common roles in the maintenance of intracellular Ca\(^{2+}\) homeostasis. An immunoprecipitation experiment showed colocalization of Cls5p (Pfy1p) with Vps16p and Vps33p in the vacuolar membrane (Xu and Wickner, 2006). The four vps mutants were originally isolated as class I vacuolar morphology (vam) mutants, with tiny or invisible vacuoles (Wada et al., 1992), whereas the vacuoles in cls5-1 mutants were fragmented in the presence of high levels of environmental Ca\(^{2+}\) (unpublished data). Therefore, it is likely that Cls5p and the four Vps proteins are involved in vacuolar biogenesis and its functions.

**Positive or negative regulation of calcineurin in functional units of Ca\(^{2+}\) homeostasis and Ca\(^{2+}\) regulatory pathways.** We examined how calcineurin is involved in the cellular response to high levels of Ca\(^{2+}\) by testing the effect of FK506 on the Ca\(^{2+}\) sensitivity of a series of cls mutants. Our results suggested that calcineurin functioned either positively or negatively with the functional CLS gene units. We confirmed that FK506 enhanced the Ca\(^{2+}\) sensitivity of the class I cls (vma) mutants, which were defective in vacuolar membrane H\(^+-\)ATPase. Several lines of evidence suggest that Ca\(^{2+}\) transport into the vacuoles of class I cls mutants is decreased (Ohya et al., 1991; Tanida et al., 1995). Yeast cells have compensatory mechanisms for Ca\(^{2+}\) transport into the vacuole, such as the Ca\(^{2+}\)-ATPase Pmc1p (Cunningham and Fink, 1994), and calcineurin positively regulates Pmc1p through activation of the transcription factor Crz1p (Stathopoulos and Cyert, 2013). Although class II cls mutants were fragility in vacuolar morphology (vam) mutants, with tiny or invisible vacuoles (Wada et al., 1992), whereas the vacuoles in cls5-1 mutants were fragmented in the presence of high levels of environmental Ca\(^{2+}\) (unpublished data). Therefore, it is likely that Cls5p and the four Vps proteins are involved in vacuolar biogenesis and its functions.

The identified clusters can be used to predict the function of the uncharacterized CLS genes. One of the examples is class VI, to which three genes, Rif4, Gly1, and YEL045C, were assigned. Rif4 is required for riboflavin biosynthesis, which is a precursor of flavin adenine dinucleotide (FAD; Oltmanns and Bacher, 1972). Gly1p is the glycine biosynthetic enzyme threonine aldolase (McNeil et al., 1994), and YEL045C is a dubious open reading frame in the promoter region of the GLY1 gene. In the filamentous fungus Ashbya gossypii, which is closely related to yeast, production of riboflavin was improved by overexpression of the GLY1 gene (Monschau et al., 1998), suggesting that Gly1p is involved in riboflavin biosynthesis. The Ca\(^{2+}\)-sensitive growth phenotype of the class VI cls mutants was suppressed by the addition of riboflavin. Although the molecular mechanism remains to be elucidated, all class VI cls mutants exhibited defects not in vacuolar acidification or sphingolipid biosynthesis, but in perturbation of the energy balance of NADPH.

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FIGURE 7: Correlation-based network of cls mutants. Similarities of Ca\(^{2+}\)-cls interaction profiles were measured for all gene pairs by calculating Pearson product–moment correlation coefficients (PCCs) from the Ca\(^{2+}\)-cls interaction matrix (PCC > 0.2) and are shown as positive (red line) or negative (blue line) correlations of the Ca\(^{2+}\)-cls interaction profile. Node size stands for the average number of significant Ca\(^{2+}\)-cls interactions within each class (p < 0.05, Wald test; Figure 5A). In addition, the effects of FK506 on each cls mutant are presented as node color (Supplemental Figure S4 and Supplemental Table S2). The node outline indicates the proportion of significant negative Ca\(^{2+}\)-cls interactions of each mutant (Figure 5B). Gene ontologies of each class were obtained from the Saccharomyces Genome Database (www.yeastgenome.org/). Relative Ca\(^{2+}\) content compared with wild type is shown in brackets. Data from Ohnuki et al. (2007) and Yoshida et al. (2013). Divalent cation sensitivity is underlined for each class (Supplemental Tables S1 and S2). Whereas all of the vma mutants showed broad divalent cation sensitivity, 29 strains showed Ca\(^{2+}\)-specific sensitivity, such as mutants of IV, VIII, and IX classes (sensitivity to other divalent cation rather than Ca\(^{2+}\)) decreases the Ca\(^{2+}\)-specific sensitivity. The inner square next to each class represents quinacrine staining (red, dark pink, light pink, and white illustrate vacuoles that were not stained, vacuoles with morphological defects, stained vacuoles, and a mixture of stained and nonstained vacuoles, respectively). The outer square displays the Pet\(^{\text{+}}\) phenotype (dark blue, light blue, and white represent minus, mixture of minus and plus, and plus Pet\(^{\text{+}}\) phenotype, respectively). Data from Ohnuki et al. (2007).

mutants, including cls2, class V CLS genes may function downstream of calcineurin. Consistent with this, the Ca\(^{2+}\)-cls interaction profiles of class I cls (vma) mutants negatively correlated with class V cls mutants, which are localized to the ER (Ohya et al., 1991; Tanida et al., 1995), suggesting that the opposing calcineurin responses may be a result of the different Ca\(^{2+}\)-cls interaction profiles.

Our results also suggested that calcineurin negatively regulates bud formation under conditions of high Ca\(^{2+}\). The Ca\(^{2+}\) sensitivity of class IV (Ca\(^{2+}\)-modulated bud formation) cls mutants was suppressed by FK506. The Ca\(^{2+}\)-cls interaction profiles of class IV cls mutants tended to negatively correlate with class I cls (vma) mutants, indicating the opposite response of calcineurin. Because elevated cytosolic Ca\(^{2+}\) has harmful effects on cellular physiology (Missiaen et al., 2000), one interesting idea is that calcineurin may function as a checkpoint that senses cytosolic Ca\(^{2+}\). On elevation of cytosolic Ca\(^{2+}\), calcineurin up-regulates Ca\(^{2+}\) sequestration to the vacuole and down-regulates bud formation to avoid the toxic effects of Ca\(^{2+}\) and maintain Ca\(^{2+}\) homeostasis. Note that Ca\(^{2+}\) stress results in distinct changes of the global gene expression pattern in yeast through the calcineurin/Crz1p signaling pathway (Yoshimoto et al., 2002). Therefore, in order to test this hypothesis, decipherment of the global gene expression in each cls mutant can be considered as a future direction.

**Study of the morphological chemical-genetic interaction profiles**

In the eukaryotic model organism S. cerevisiae, the nonessential gene deletion set (Winzeler et al., 1999) and recently developed libraries of essential genes harboring conditional and hypomorphic alleles (Mnaimneh et al., 2004; Ben-Aroya et al., 2008; Jin et al., 2011; Li et al., 2011) have enabled rapid and comprehensive identification of factors that function in cellular processes of interest. However, functional characterization of these genes, which generally requires gene-by-gene follow-up investigation, remains a bottleneck. Our chemical-genetic interaction approach allows systematic characterization of individual genes, providing a systems-level global view, as well as the molecular bases for cellular processes. Chemical-genetic interaction profiling based on high-dimensional morphological phenotypes described here identifies critical relationships within pathways with multiple inputs (e.g., environmental stress, chemical perturbation, gene perturbation) and outputs (e.g., homeostasis, gene expression, cell cycle progression). We propose that this strategy will be applicable for the systematic analysis of complex cellular processes.

**MATERIALS AND METHODS**

**Strains and growth conditions**

Strains are listed in Supplemental Table S3. All strains are isogenic derivatives of BY4741 (MATa; his3; leu2; met15; ura3; Euroscarf, www.euroscarf.de). Two mutants, cls4-1 and cls5-1, were constructed from YOC138-C (MATa ade1 cls4-1; Ohya et al., 1986a) and YOC989 (MATa leu2 lys2 trp1 ura3 cls5-1; Yoshida et al., 2013). Briefly, a kanamycin-resistance cassette was amplified from the plasmid pF6A6a-GFP(S65T)-kanMX6 (Longtine et al., 1998), targeted to the 3’-untranslated region of the CLS4 and CLS5 genes, and located to a backbone downstream of the stop codon. The cls4-1 and cls5-1 loci linked to the kanamycin-resistance cassette were amplified by PCR, the amplified fragments were transformed into BY4741, and...
Geneticin-resistant transformants were selected. Replacement of the wild-type CLS4 and CLS5 genes was confirmed by PCR and sequencing.

S. cerevisiae strains were grown in YPD medium with 1% (wt/vol) Bacto yeast extract (BD Biosciences, San Jose, CA), 2% (wt/vol) polypeptone (Wako Chemicals, Richmond, VA), and 2% (wt/vol) dextrose, pH 5.5. Ca\(^{2+}\) sensitivity was tested using 100 mM CaCl\(_2\) (Ca\(^{2+}\)-rich medium). YPG medium (1% Bacto yeast extract, 2% polypeptone, 2% [vol/vol] glycerol) was applied to survey Pet\(^+\) phenotypes (inability to grow on a nonfermentable carbon source). Sensitivity to other divalent cations, including 100 mM MgCl\(_2\), 3 mM MnCl\(_2\), and 3 mM MnCl\(_2\), was also surveyed. Agar (2% [wt/vol]) was used as the solidifier. YPD medium contains 0.4 \(\mu\)g/ml FK506 (Cayman Chemical, Ann Arbor, MI), and various concentrations of CaCl\(_2\) (0, 25, 50, 75, and 200 mM) were used to examine the effects of FK506 on cell growth under conditions of high Ca\(^{2+}\). Yeast growth was computationally analyzed via image quantification using ImageJ software, version 1.50i (Abramoff et al., 2004). One-way analysis of variance (ANOVA) with a negative-binomial probability distribution function was used for statistical analysis. Results were also visually confirmed. To check riboflavin effect on Ca\(^{2+}\)-sensitivity, a higher CaCl\(_2\) concentration (300 mM; compare with the main experiment, 100 mM) was used to detect any slight effects on suppression of Ca\(^{2+}\)-sensitive growth.

**Measurements of exchangeable and nonexchangeable Ca\(^{2+}\) pools**

Intracellular calcium content was measured as described previously (Cunningham and Fink, 1994) with minor modification. Briefly, exponentially growing yeast cells ([1–1.5] \(\times\) 10\(^7\) cells/ml) in YPD medium, pH 5.5, were collected, resuspended in YPD medium, pH 5.5, containing \(40\)CaCl\(_2\) (>10 Ci/g, PerkinElmer, Waltham, MA), and incubated at 30°C for 6.5 h. Protein content was measured using a bichinonic acid protein assay kit (Pierce, Rockford, IL) after incubation of each strain in the absence of \(40\)CaCl\(_2\) in the same conditions as \(45\)CaCl\(_2\)-treated cells. Cultured yeast cells were collected and suspended in 0.2 ml of 10% trichloroacetic acid.

**Acquisition and image processing**

Ca\(^{2+}\) treatment of yeast cells was performed as described previously (Ohnuki et al., 2007). Cells in log phase (8 \(\times\) 10\(^6\) cells/ml) in YPD medium were collected, washed once in YPD medium with or without 100 mM CaCl\(_2\), and resuspended in 4 ml of the respective medium at a final concentration of 2 \(\times\) 10\(^6\) cells/ml. Cells were then incubated for 5 h at 30°C, washed once with YPD medium, fixed in YPD medium supplemented with 3.7% formaldehyde, and resuspended in phosphate-buffered saline (PBS; 0.1 M potassium phosphate buffer, pH 6.5). Triple staining and image analysis using CalMorph (version 1.0) software was performed as previously described (Ohya et al., 2005). CalMorph automatically calculated 501 morphologic parameters of each yeast cell. Cells were stained for three components: fluorescence isothiocyanate–concanavalin A (Sigma-Aldrich, St. Louis, MO), rhodamine–phalloidin (Invitrogen, Carlsbad, CA), and 4',6-diamidino-2-phenylindole (Sigma-Aldrich), which were used to stain mannoprotein, actin, and the nucleus, respectively. Images of triple-stained cells were captured using an Axiosimager M1, a 100X EC-Plan NEO objective lens (Carl Zeiss) equipped with a CoolSNAP HQ cooled charge-coupled device camera (Roper Scientific), and AxioVision software (Carl Zeiss).

**Statistical model to assess Ca\(^{2+}\)–cls interactions**

Statistical analyses were performed with R software (www.r-project.org). To statistically assess Ca\(^{2+}\)–cls interactions for morphological phenotypes, a GLM, an extension of the normal linear model, was used, which applied not only a Gaussian, but also additional probability distributions (Nelder and Wedderburn, 1972). Models of the probability distributions for the 501 morphological parameters were determined to accommodate the statistical model used in the GLM as previously described, with some modifications (Yang et al., 2014). Of the 501 parameters calculated by CalMorph, 220 parameters were coefficients of variation (CVs) of their related mean parameters. Estimations of cell-to-cell independent variability were obtained via nonlinear Lowess regression of the CV values as previously described (Yvert et al., 2013) to uncouple concomitant dependence between the CV parameters and the mean parameter values (Levy and Siegal, 2008). After normalization, 220 CV parameters were assumed to be Gaussian-distributed. In addition, 183 parameters, representing the mean cell morphologies with positive continuous values, were assumed to be gamma-distributed, as previously described (Yang et al., 2014). Moreover, 37 parameters, which represented the mean cell morphologies with continuous values ranging from 0 to 1, were assumed as beta- or zero-inflated beta-distributed. The remaining 61 parameters, which represented the ratio of cells in the specimen, were assumed as binomial- or beta-binomial-distributed with or without overdispersion, respectively.

The ANCOVA model, which is a blend of ANOVA and regression in the multiple linear model, was applied for assessment of Ca\(^{2+}\) treatment and mutation effects on the cells regarding Gaussian, gamma, beta, zero-inflated beta, binomial, and beta-binomial parameters in the manner of the GLM. The statistical model was defined as follows:

\[
\eta(y_i) = \beta_0 + \beta_1x_i + \beta_2d_i + \beta_3x_i \cdot d_i + \beta_4e_i + \epsilon_i
\]

where \(\eta\) is the link function, \(y_i\) is a response variable (parameter values), \(\beta_0\) is the intercept, \(\beta_1\) is a fixed effect of Ca\(^{2+}\) treatment, \(x_i\) is a concentration of Ca\(^{2+}\) treatment as an explanatory variable for \(y_i\), \(\beta_2\) is a fixed effect of the cls mutation, \(d_i\) is an indicator of the mutation as an explanatory variable, \(\beta_3\) is a fixed effect of the Ca\(^{2+}\)–cls interaction, of which the explanatory variable \(x_i \cdot d_i\) of the interaction term was defined as a product of \(x_i\) and \(d_i\) in the linear model, \(\beta_4\) is a fixed effect of the confounding factor, \(e_i\) is an indicator of the confounding factor as an explanatory variable, and \(\epsilon_i\) is the error. The best model was selected from the combination of probability distributions and the linear models (e.g., zero-inflated beta vs. beta, binomial vs. beta-binomial, with or without confounding factors) for each parameter based on the Akaike information criterion (AIC; Akaike, 1998). Likelihood ratio test for the interaction term was carried out to detect parameters with significant Ca\(^{2+}\)–cls interactions among sets of cls mutants (\(p < 0.05\)). The models of probability distributions, corresponding link functions, and descriptions of analyzed parameters are listed in Supplemental Tables S4 and S5, respectively. The Z value, calculated by the Wald test for \(\beta_1\) of maximum likelihood estimation in each parameter, was used as a degree of Ca\(^{2+}\)-induced morphological change of wild-type (his3Δ or YPH499) cells in this study (referred to as \(Z_{\text{wt}}\) in the text and figures).
The Z value, calculated by the Wald test for $\beta_1$ of the maximum likelihood estimation in each cls mutant, was used as a degree of the Ca$^{2+}$–cls interaction. The $\pi$ score as a phenotypic interaction score was calculated as follows:

$$
\pi = \begin{cases} 
\beta_3 & \text{if } (\beta_1 < 0 \text{ and } \beta_3 > 0) \text{ or } (\beta_1 > 0 \text{ and } \beta_3 < 0) \\
-\beta_3 & \text{else if } (\beta_1 > 0 \text{ and } \beta_3 < 0) \text{ or } (\beta_1 < 0 \text{ and } \beta_3 > 0)
\end{cases}
$$

A linear model of dispersion in each parameter was selected from two models by AIC to detect interaction between cls mutation and Ca$^{2+}$ treatment in Figure 5, where one of the models was a null model and the other was a linear combination of experimental conditions (each Ca$^{2+}$ concentration in each strain). Of the 501 parameters, 247 were selected to have different dispersion among the experimental conditions by AIC. At $p < 0.05$ by likelihood ratio test, 245 of 501 parameters were detected to have significant interaction(s). Of the 245 parameters, 113 were detected to have significant Ca$^{2+}$ effects on wild type at $p < 0.05$ by Wald test. Among 7006 assays (62 cls mutants $\times$ 113 parameters), 1612 combinations were detected to have significant interaction at $p < 0.05$ by Wald test as shown in Figure 5, where FDR was estimated as 0.24 by randomization with >2000 iterations.

**PCA and hierarchical cluster analysis**

To extract the independent morphological features, PCA was performed using morphological data of a null distribution (Ohnuki et al., 2012) with minor modification. The 209 Z values calculated from the parameter values of the 122 replicated wild-type data set (null-distributed data) were subjected to PCA based on the correlation matrix. First, 19 PCs reached 70% of the cumulative contribution ratio (CCR). Significantly correlated parameters with each PC ($p < 0.01$ after Bonferroni correction) were selected and are listed in Supplemental Figure S1.

A similar approach was performed for the identification of independent morphological features correlated with FKS06 effects. The independent morphological features were extracted as previously described (Ohnuki et al., 2012). The alleviating and aggravating effects of FKS06 on cell growth under conditions of high Ca$^{2+}$ were particularly reflected in the first and second PCs (Supplemental Figure S7B). We found that 67 and 7 parameters were significantly correlated with PC1 and PC2, respectively, at $p > 0.60$ of the absolute value of loadings, which is equivalent to the correlation coefficient between the PC score and the $z$ score ($p < 5 \times 10^{-7}$ after Bonferroni correction, Student’s $t$ test). In the second PCA, the 67 and the 7 parameter values for PC1 and PC2, respectively, of the 122 replicated wild-type data set (null-distributed data) were used. Parameters with $>0.70$ of absolute value of the loadings for each PC were selected, and the parameters that correlated with the first five PCs (PC1–a–e) and the first three PCs (PC2a–c) are listed in Supplemental Figure S8.

Hierarchical clustering analysis (HCA) for the 62 cls mutants was performed as previously described (Ohnuki et al., 2007), with minor modification. Briefly, to exclude correlation among the morphological parameters, PC scores of the Ca$^{2+}$–cls interaction profiles were applied. HCA based on the dissimilarity defined by 1 – R (Pearson product–moment correlation coefficient) was applied to the 16 PC scores (80% of the CCR). Clusters were assessed using the R package pvclust tool at AU $p > 0.95$ (Suzuki and Shimodaira, 2006).

**Quinacrine staining**

For quinacrine labeling, exponentially growing cells were harvested by centrifugation and washed three times with 50 mM sodium phosphate buffer, pH 7.5, containing 2% glucose. Quinacrine was added at a final concentration of 500 µM in 50 mM sodium phosphate buffer, pH 7.5, containing 2% glucose. After a 10-min incubation at 25°C, the cells were collected by centrifugation, washed three times with YPD, pH 7.5, and examined by fluorescence microscopy with a blue filter.

**In vivo [$^{3}$H]palmitic acid labeling**

Cells were collected in log phase, resuspended in YPD medium (1.0 A$\text{OD}_{600}$ unit/ml), and labeled with 2.5 µCi of [$^{3}$H]palmitic acid (American Radiolabeled Chemicals, St. Louis, MO) for 90 min at 30°C. Cells were collected by centrifugation and suspended in extraction buffer (ethanol, water, diethyl ether, pyridine, and 15 N ammonia [15:15:5:1:0.018, vol/vol]). After 15-min incubation at 60°C, extracted lipids were separated from cell debris by 2-min centrifugation at 2000 × g. Lipids in the cell debris were reextracted using extraction buffer. Radioactivity of the lipid solution was measured, and samples of equal radioactivity were used for further analysis. Each lipid solution was alkali treated by incubating with 0.2x volume of 0.5 N NaOH in methanol for 40 min at 37°C and then neutralized with acetic acid. Lipids were dried and suspended in 100 µl of water-saturated butanol. Then 50 µl of water was added, vigorously mixed, and separated into two phases by centrifugation at 9000 × g for 1 min. The butanol phase was collected, and lipids in the water phase were reextracted by adding 100 µl of water-saturated butanol. The two butanol phases were pooled together, dried, and suspended in 20 µl of chloroform/methanol/water (5:4:1 [vol/vol]). Lipids were separated by TLC on Silica Gel 60 high-performance TLC plates (Merck, Whitestation, NJ) with chloroform, methanol, and 4.2 N ammonia (9:7.2 [vol/vol]) as the solvent system.

**NADP$^{+}$ measurement**

Cells were grown in YPD to log phase, and each culture of ~108 cells was sampled. Harvested cells were washed with ice-cold PBS and PBS plus 0.01% Triton X-100 and subjected to extraction and detection with Fluoro NADP$^{+}$/NADPH (Cell Technology, Fremont, CA) according to the manufacturer’s instructions, with minor modification. Cells were disrupted with glass beads in lysis buffer and heated at 60°C for 30 min and cooled on ice. Fluorescence was measured at the excitation and emission wavelengths of 540 and 590 nm, respectively, with a Shimadzu RF-5300PC spectrophotofluorometer.

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**REFERENCES**


