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The high-affinity cAMP phosphodiesterase of *Saccharomyces cerevisiae* is the major determinant of cAMP levels in stationary phase: involvement of different branches of the Ras–cyclic AMP pathway in stress responses

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Abstract

The Ras–cyclic AMP (cAMP) pathway is a major determinant of intrinsic stress resistance of the yeast *Saccharomyces cerevisiae*. Here, we isolated *IRA2*, encoding the Ras GTPase activator, as a global stress response gene. Subsequently, we studied the other negative regulators on the separate branch of the Ras–cAMP pathway, the low- or high-affinity cAMP phosphodiesterase encoded by *PDE1* or *PDE2*, respectively. Deletion of *PDE2*, similar to *ira2* deletion, rendered cells sensitive to freeze–thawing, peroxides, paraquat, cycloheximide, heavy metals, NaCl, heat, or cold shock. However, deletion of *PDE1* did not affect stress tolerance, although it exacerbated stress sensitivity caused by the *pde2* deletion, indicating that *PDE1* can partly compensate for *PDE2*. Deletion of *IRA2* uniquely led to high sensitivity to cumene hydroperoxide, suggesting that *IRA2* may have a distinct role for the response to this stress. Stress sensitivity of yeast cells in general correlated with the basal level of cAMP. Interestingly, yeast cells lacking *PDE2* maintained higher cAMP levels in stationary phase than exponential growth phase, suggesting that Pde2p is the major regulator of cAMP levels in stationary phase. Depletion of Ras activity could not effectively suppress stress sensitivity caused by lack of cAMP phosphodiesterases although it could suppress stress sensitivity caused by lack of *IRA2*, indicating that cAMP accumulation in stationary phase can be mediated by other signaling proteins in addition to Ras. Our study shows that control of cAMP basal levels is important for determining intrinsic stress tolerance of yeast, and that the cAMP level during stationary phase is a result of a dynamic balance between its rates of synthesis and degradation.

Keywords: Ras–cAMP pathway; *PDE1*; *PDE2*; *IRA2*; Stress response; *Saccharomyces cerevisiae*

Survival of a cell in a stress situation is dependent on its ability to respond appropriately to alterations in the environment. It has been noted that cells in stationary phase show increased stress tolerance, which appears to be an outcome of adaptation processes to nutrient deficiency [1]. This indicates that stress defence systems are in general responding to nutrient levels, and that a signal transduction pathway is required for sensing and transmitting nutrient signals to activate stress defence mechanisms. In the yeast *Saccharomyces cerevisiae*, the Ras–cyclic AMP (cAMP) pathway may be a mediator of this process. Indeed, the Ras–cAMP pathway is involved in a variety of stress responses. For example, the Ras–cAMP pathway can regulate cellular

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responses to the STRE-mediated stress [2–6], the Pos9/Skn7-mediated oxidative stress [7], hyperosmotic stress [8,9], and freezing and thawing [10,11].

Ras proteins, shuttling between the GDP and GTP-bound forms, play diverse physiological roles by mediating signaling for receptor protein kinases and tyrosine kinase-associated receptors. Uniquely, Ras proteins in yeast (encoded by *RAS1* and *RAS2*) are associated with adenylate cyclase (encoded by *CYR1*/*CDC35*) and regulate the level of cAMP that functions exclusively to activate cAMP-dependent protein kinase A (PKA) [12,13]. PKA is a heterotetramer of two regulatory subunits encoded by *BCY1* and two catalytic subunits redundantly encoded by *TPK1*, *TPK2*, and *TPK3* [14]. cAMP binds to the regulatory subunits of PKA and induces dissociation of the catalytic subunits. PKA phosphorylates various target proteins involved in transcription, energy metabolism, cell cycle progression, and accumulation of glycogen and trehalose [3,15–22].

The efficacy of the Ras–cAMP pathway is determined by the level of cAMP. cAMP levels can be regulated at the level of its synthesis (the Ras-adenylate cyclase module) and degradation (the cAMP phosphodiesterases). The highly homologous Ira1p and Ira2p downregulate Ras activity by stimulating the GTPase activity of Ras, while Cdc25p, the guanine nucleotide exchange factor, activates Ras [23–26]. The cAMP phosphodiesterases, encoded by *PDE1* or *PDE2*, inhibit PKA by hydrolyzing cAMP [27,28]. These enzymes share no sequence homology, and have different affinity to cAMP; Pde2p has higher affinity ($K_m = 0.2\ \mu M$) than Pde1p ($K_m = 3\ \mu M$) [28–30]. Deletion of *PDE1*, but not *PDE2*, results in a much higher cAMP accumulation upon stimulation with reagents inducing intracellular acidification, indicating a specific role of the low-affinity cAMP phosphodiesterase in an agonist-induced cAMP signaling [31]. The intracellular cAMP level transiently increases upon addition of fermentable carbon sources [32], and both Pde1 and Ira proteins are required for this process [25,28]. Meanwhile, yeast cells are known to maintain low basal levels of cAMP in stationary phase [33], which may be important for relatively high stress tolerance in the growth phase. However, it is not clear yet how the regulatory components of the Ras–cAMP pathway are involved in maintaining the basal level of cAMP in stationary phase.

Previously, we have shown that the Ras–cAMP pathway is essential for survival of yeast cells following freezing and thawing [10]. In the present study, we have further studied the involvement of the Ras–cAMP pathway in stress responses. Specifically, we isolated *IRA2* and *PDE2* as important genes for stress defence, and studied the involvement of these negative regulators of the Ras–cAMP pathway in determining the intrinsic stress resistance. We also show that stress sensitivity is correlated with basal cAMP levels, and that the basal cAMP level in stationary phase is most affected by the high-affinity cAMP phosphodiesterase.

Materials and methods

Strains, media, and transformation. Yeast strains used are described in Table 1. Strain Ras2(II) was made by back-crossing JC302-26B [34] to CY4 [35] four times. Strains, unless otherwise specified, were grown at 30 °C, with shaking at 180 rpm in 3 ml medium in a 16 × 100-mm culture tube. YEPD medium contained 2% glucose, 2% bactopeptone, and 1% yeast extract and SD medium contained 2% glucose, 0.17% yeast nitrogen base (Difco), 0.5% ammonium sulfate (Oxoid) and auxotrophic requirements at 40 mg/L where necessary. Media were solidified by adding 2% agar. Transformation of yeast cells was carried out with lithium acetate [36].

Gene deletion and confirmation. Genes were deleted using a PCR-based direct gene deletion technique [37]. Primers used for gene disruption were TGGTTGTATTCGAAATAACTATACTTGGGGC CAATGGAGGACCCACCGAAGCAGATTGTACTGAGAGTGC and ATTATAGAAACAAAGTGTGGCCTTCTAGCGCAATAGA TATTCTCAAGTCTGCATCTGTGCGGTATTTCACAC for *PDE1* deletion, TGTCCACCCTTTTTCTGATTGGAATACACGAGATT GAGAAATCTCAAACAGCAGATTGTACTGAGAGTGC and TG CTATTGTGGTTTCTTGTGTTTCATCCAGTATTCTTTATTGA TTTTGACGCATCTGTGCGGTATTTCACAC for *PDE2* deletion, and ATTTTTTGATATCAACTAAACTGTATACATTATCTTTCT TCAGGGAGAAGCCGGCATCAGAGCAGATTGTA and ATGC TTACAGATAGATATTGATATTTCTTTCATTAGTTTATGTAA CACCTCGCATCTGTGCGGTATTTCACA for *IRA2* deletion. PCR was performed using the single copy plasmid pRS414 as the template to prepare *PDE1* disruption DNA, and pRS413 for *PDE2* or *IRA2* disruption. Successful disruption was confirmed by PCR using primers designed from the upstream of target DNA and from the internal region of disruption markers. Primers used for confirmation were ACAAGACGTTGATCCTGATCT and GGAATCTAGAGCACAT TCTGCGGCC (*TRP1* primer) for *PDE1* deletion, AGAAGACAT CATTGCTGGCTTC and CATGTATCATATGGTCCAGAAACCC (*HIS3* primer) for *PDE2* deletion, and CTTGGACCTCTAG AACCGATGT and *HIS3* primer for *IRA2* deletion. The *pde1 pde2* double-deletion mutant was made by crossing the *pde1* and *pde2* mutants, and subsequent dissection of the diploid strain. All deletions were verified as single mutations by back-crossing each mutant strain to its parental wild-type strain and measuring the segregation of phenotypes.

Plasmids. YEp13*PDE2* was cloned by screening a yeast genomic DNA library (ATCC37323) in the *fsm1* mutant Fsm4C. It contains DNA from chromosome XV spanning 1,011,721–1,015,679 bp where *PDE2* is the only intact open reading frame. pSEY18*YAP1* and YEp24*IRA2* [25] were donated by W.S. Moye-Rowley and J. Cannon, respectively.

cAMP determination. cAMP samples were prepared as previously described [28]. An appropriate volume of cell culture was harvested and washed in 20 mM potassium phosphate buffer (pH 7.0). Cells were homogenized in 5% (w/v) TCA with glass beads. Supernatants were collected, washed with water-saturated diethyl ether, and freeze-dried. cAMP was measured using an enzyme-immunoassay system (Amersham) as indicated by the manufacturer.

Freezing and thawing conditions. Cells were harvested by centrifugation, washed in 0.1 M sodium phosphate buffer (pH 7.0), and suspended to an A_{600} of 3 in the same buffer. Aliquots (0.3 ml) of cells were transferred to thin-walled 1.5-ml polycarbonate tubes (Greiner Labortechnik), frozen at −20 °C for 2 h, and thawed at room temperature for 15 min as previously described [10]. Survival was determined by diluting cells into YEPD medium at room temperature and

Table 1
Yeast strains used for this study

Strains	Genotype	Source
CY4	*MAT***a** *ura3-52 leu2-3, 112 trp1-1 ade2-1 his3-11 can1-100*	[35]
CY29	As in strain CY4 but *yap1Δ::HIS3 fsm1*	[35]
JY29	As in strain CY4 but *yap1Δ::HIS3*	[41]
Fsm4C	As in strain CY4 but *fsm1*	This study
Jpde1	As in strain CY4 but *pde1Δ::TRP1*	This study
Jpde2	As in strain CY4 but *pde2Δ::HIS3*	This study
Jpde12	As in strain CY4 but *pde1Δ::TRP1 pde2Δ::HIS3*	This study
Jira2	As in strain CY4 but *ira2Δ::HIS3*	This study
Fsmpde1	As in strain CY4 but *pde1Δ::TRP1 fsm1*	This study
Fsmpde2	As in strain CY4 but *pde2Δ::HIS3 fsm1*	This study
CY4D	*MAT***a**/*MATα ura3-52/ura3-52 leu2-3, 112/leu2-3, 112 trp1-1/trp1-1 ade2-1/ade2-1 his3-11/his3-11 can1-100/can1-100*	This study
CY4/29	As in strain CY4D but *YAP1/yap1Δ::HIS3 IRA2/fsm1*	This study
CY29D	As in strain CY4D but *yap1Δ::HIS3/yap1Δ::HIS3 fsm1/fsm1*	This study
CY4/pde2	As in strain CY4D but *PDE2/pde2Δ::HIS3*	This study
CY4/fsm	As in strain CY4D but *IRA2/fsm1*	This study
CY4/ira2	As in strain CY4D but *IRA2/ira2Δ::HIS3*	This study
Fsm/ira2	As in strain CY4D but *fsm1/ira2Δ::HIS3*	This study
Fsm/pde2	As in strain CY4D but *PDE2/pde2Δ::HIS3 fsm1/IRA2*	This study
JC302-26B	*MATα ura3-52 leu2 his4-539 ras2Δ::LEU2*	[34]
Ras2(II)	*MAT***a***ade2 leu2 ura3 his3 ras2Δ::LEU2*	This study
Fsm/ras2(II)	*MAT***a**/*MATα ura3/ura3 leu2/leu2 trp1/TRP1 ade2/ade2 his3/his3 RAS2/ras2Δ::LEU2 fsm1/IRA2*	This study
Pde12/ras2(II)	As in strain Fsm/ras2(II) but *pde1Δ::TRP1/PDE1 pde2Δ::HIS3/PDE2 RAS2/ras2Δ::LEU2*	This study
LRA85	*MAT***a***his4 leu2 ura3-52 cdc35-11*	K. Tatchell
Fsm/LRA85	*MAT***a**/*MATα his3/HIS3 his4/HIS4 leu2/leu2 ura3/ura3 CDC35/cdc35-11 fsm1/IRA2*	This study
MB12	*MAT***a** *ade2 can1 his3 leu2 trp1 ura3 tpk1 Δ::LEU2 tpk2Δ::HIS3*	[42]
Wmsn2msn4	*MAT***a** *ade2 can1 his3 leu2 trp1 ura3 msn2-3::HIS3 msn4-1::TRP1*	[54]
Fsm/MB12	*MAT***a**/*MATα ade2/ade2 can1/can1 his3/his3 leu2/leu2 trp1/trp1 ura3/ura3 TPK1/tpk1Δ::LEU2 TPK2/tpk2Δ::HIS3 fsm1/IRA2*	This study
dbcy1	*MAT***a** *his3 leu2 trp1 ura3 ade8 can1 suc2 ade2 can1 bcy1Δ::LEU2*	M. Yamamoto

plating on YEPD plates. Cells were grown at 30 °C for 2 days before colony counting.

Assay for stress resistance on plate. Five microliters of cell culture, diluted to an A_{600} of 0.1, or 1 in fresh YEPD medium, was spotted onto YEPD plates containing 3 mM H_2O_2, 0.7 mM *tert*-butyl hydroperoxide (*t*BHP), 0.1 mM cumene hydroperoxide (CHP), 0.5 mM diamide, 2.5 mM paraquat, 0.25 μg/ml cycloheximide, 0.05 mM $CdSO_4$, 1 mM $CoCl_2$, or 1.5 M NaCl, and incubated at 30 °C for visualization of phenotype. Alternatively, cells were replica plated onto the test plates. For heat or cold-shock test, cells were spotted onto YEPD plates and incubated at 37 or 14 °C.

Results

Isolation of IRA2 as a gene required for global stress defence

While maintaining the strain CY29 [35], a deletion mutant of *YAP1* that encodes a transcription factor for oxidative stress defence [38–40], we isolated a colony that was highly sensitive to freezing and thawing relative to the isogenic wild-type strain CY4 (Fig. 1A). This was interesting since the Yap1 transcription factor is not essential for yeast cell survival after freeze–thaw stress [41]. Overexpression of *YAP1* in CY29 did not restore freeze–thaw tolerance, and JY29, another *yap1* mutant on an isogenic background, displayed a wild-type level of freeze–thaw tolerance (Fig. 1A), indicating that the freeze–thaw sensitivity of CY29 was not due to the absence of *YAP1*. Subsequently, we crossed CY29 to CY4 and analyzed the resulting heterozygous diploid cells. The heterozygous diploid CY4/29 did not show freeze–thaw sensitivity (Fig. 1A), and tetrad analysis of asci from CY4/29 displayed 2:2 segregation of the freeze–thaw sensitive phenotype (data not shown), indicating that the freeze–thaw sensitive phenotype of CY29 was caused by a single recessive mutation. This mutation was provisionally named *fsm1* (freeze–thaw sensitive mutation 1). In addition to the freeze–thaw sensitivity, the *fsm1* mutant was sensitive to oxidative stresses induced by H_2O_2, tBHP, CHP, the superoxide generator paraquat, and diamide, and other types of stress induced by cycloheximide, NaCl, cobalt, cadmium, heat shock at 37 °C, and cold shock at 14 °C (Fig. 1B). Compared to the *yap1* deletion, which elicited sensitivity only to peroxides, cycloheximide, and heavy metals, the *fsm1* mutation broadly affected a cell's ability for stress resistance, indicating that *FSM1* is a crucial gene for stress response.

In attempts to clone *FSM1*, we screened genomic DNA libraries under various stress conditions listed above. Regardless of the conditions, *PDE2* was isolated as a major suppressor of the *fsm1* phenotype, conferring

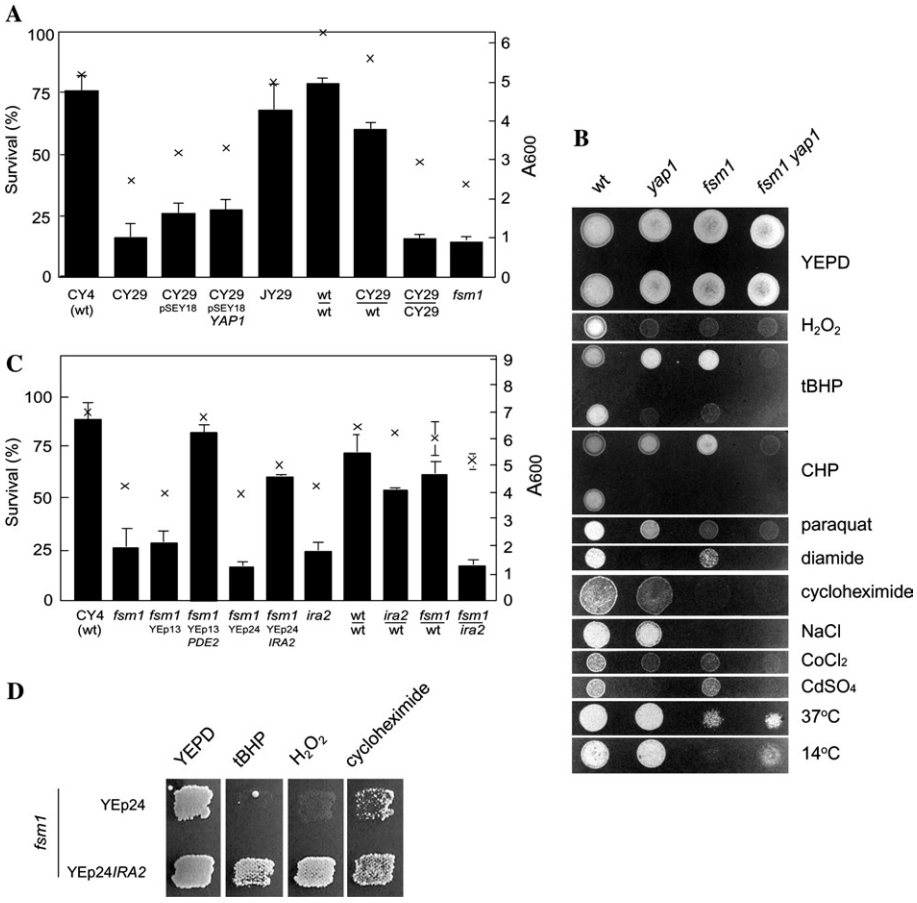

Fig. 1. Isolation of *IRA2* as a stress defence gene. (A) Freeze–thaw tolerance of strains grown on YEPD medium for 2 days was determined. CY4 is the wild-type strain for CY29, JY29, and Fsm4C (*fsm1*). CY4D (wt/wt) is the homozygous diploid of CY4 and the wild-type for CY4/29 (CY29/wt) and CY29D (CY29/CY29). Percentage survival (column) is expressed relative to the culture viability immediately prior to freezing (%). (×) Indicates A_{600} of each culture at the time points. The data shown are means (±SE) from a representative experiment performed in triplicate. (B) Strains were grown on YEPD medium for 1 day prior to plate test. Stress resistance was determined by spotting 5 μl of each diluted fraction (A_{600} of 1.0 or 0.1) onto YEPD plates containing each of the agents indicated. (C) Freeze–thaw tolerance of strains grown on YEPD medium for 2 days was determined. The *ira2* deletion mutant is isogenic to CY4. The *ira2*/wt, *fsm1*/wt, and *fsm1*/*ira2* mutant diploid strains are isogenic to CY4D (wt/wt). The data shown are means (±SE) from a representative experiment performed in triplicate. (D) The *fsm1* mutant strain Fsm4C was transformed with YEp24*IRA2*, and stress resistance of 1 day culture was determined by replica plating onto YEPD plates containing the agents indicated. Experiments were repeated at least three times with similar results.

increased growth ability and freeze–thaw tolerance (Fig. 1C). Deletion of *PDE2* also rendered cells freeze–thaw sensitive (Fig. 2A); details on the stress responses of *pde2* deletion mutants are described below. However, *PDE2* was not *FSM1* since the *fsm1* phenotype could be complemented by crossing the *fsm1* mutant to the *pde2* deletion mutant (data not shown). Next, we attempted to locate *fsm1* by genetic mapping. The *fsm1* mutation was mapped between *GSH2* (PD:NPD:T = 32:1:32, about 29 cM) and *COQ3* (PD:NPD:T = 41:0:6, about 6 cM), where we found *IRA2* at approximately 23 cM distant to *GSH2*. *IRA2* was a valid candidate for further investigation since *IRA2* encodes a Ras-GTPase activator protein, and *PDE2* was the major outcome of our screening. Overexpression of *IRA2* conferred stress tolerance to the *fsm1* mutant (Figs. 1C and D), whereas deletion of *IRA2* rendered yeast cells stress sensitive (Figs. 1C and 2). The heterozygous diploid cells made by crossing the *fsm1* mutant to the *ira2* deletion mutant were still stress sensitive (Fig. 1C for freeze–thaw response, data not shown for other types of stress responses), and tetrad analysis of asci from the diploid displayed only parental ditype segregation of *fsm1* and *ira2* (PD:NPD:T = 50:0:0). These data indicated that *fsm1* is a mutant allele of *IRA2*.

This identification was an extension of our previous finding that the Ras–cAMP pathway is important for yeast cell survival after freeze–thaw stress [10], and led to further investigation of a global stress defence mechanism in yeast mediated by the Ras–cAMP signal transduction pathway.

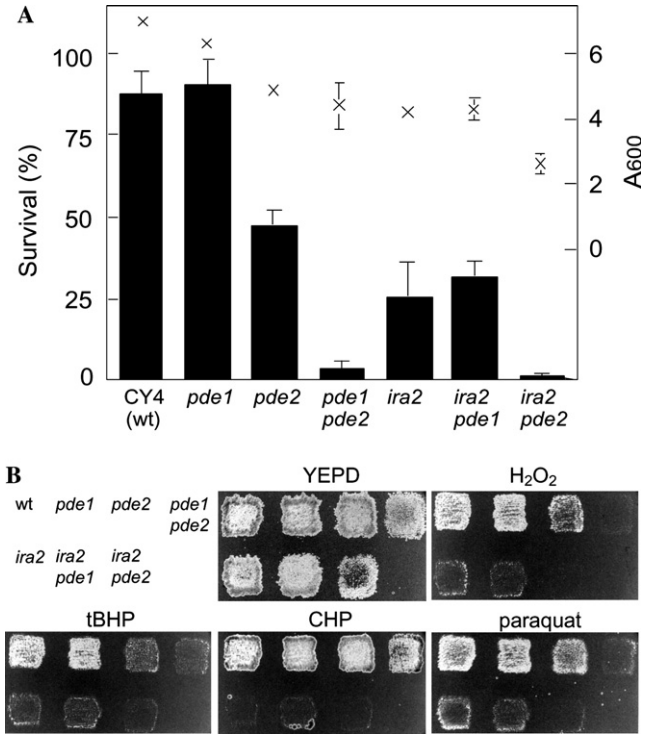

Fig. 2. The involvement of cAMP phosphodiesterases in stress response. Strains were grown on YEPD medium. CY4 is the wild-type strain of *pde1*, *pde2*, *pde1 pde2*, *ira2*, *ira2 pde1*, and *ira2 pde2* deletion mutants. (A) Freeze–thaw tolerance of strains grown on YEPD medium for 2 days was determined. Percentage survival (column) is expressed relative to the culture viability immediately prior to freezing (%). (×) Indicates A_{600} of each culture at the time points. The data shown are means (±SE) from a representative experiment performed in triplicate. (B) Stress resistance of 1 day culture was determined by replica plating onto YEPD plates containing the agents indicated. Experiments were repeated at least three times with similar results.

cAMP phosphodiesterases are also required for determination of intrinsic stress resistance: comparison of stress responses mediated by IRA2 or PDE2

Since the Ras–cAMP pathway is bifurcated at the metabolism of cAMP, and *IRA2* is a negative regulator working upstream of the synthesis of cAMP (i.e., on the Ras-adenylate cyclase branch), we were interested in the break down of cAMP mediated by cAMP phosphodiesterases. The cAMP phosphodiesterases, encoded by *PDE1* and *PDE2*, can be viewed as regulators of cAMP level in yeast cells and it is therefore possible that these two systems perform different regulatory roles for the Ras–cAMP pathway under different stress situations. To investigate this, *PDE1* and *PDE2* were deleted both singly and doubly in the wild-type strain CY4. Deletion of *PDE2* made cells sensitive to freeze–thawing (Fig. 2A) and oxidative stress induced by H_2O_2, tBHP, CHP, or paraquat (Fig. 2B), whereas deletion of *PDE1* did not affect cellular stress resistance. However, deletion of *PDE1* exacerbated stress sensitivity of the *pde2* mutant, indicating that *PDE1* can partly compensate for the absence of *PDE2*.

We next investigated whether cAMP phosphodiesterases or *IRA2* could have distinct roles in stress responses. We compared stress sensitivity of the mutants lacking cAMP phosphodiesterases or *IRA2* under different stress conditions. The *ira2* mutant and the *pde2* mutant showed, in general, a similar pattern of stress sensitivity to freeze–thawing, H_2O_2, tBHP, and paraquat while the *pde1 pde2* double-deletion mutant showed extreme stress sensitivity (Figs. 2A and B). Interestingly, the *ira2* mutant was uniquely sensitive to CHP, even more than the *pde1 pde2* mutant, indicating that *IRA2* may have a distinct role in the CHP-induced stress response. Deletion of *PDE2* exacerbated the phenotype of the *ira2* mutant while deletion of *PDE1* showed no additive effects. Since these negative regulators of the Ras–cAMP pathway can affect cAMP levels, we investigated the correlation between stress tolerance and cAMP levels in these mutants.

Correlation between intrinsic stress tolerance and basal cAMP levels

The basal level of cAMP was measured at an early exponential growth phase (A_{600} of 1) and after cells reached stationary phase (Table 2). At the early exponential growth phase in YEPD, the wild-type strain and the *pde1* mutant showed no difference in the intracellular cAMP level, whereas the *pde2* and the *ira2* mutants showed increased cAMP levels (about twofold in both cases) relative to the wild-type cells. The highest level was observed in the *pde1 pde2* double-deletion mutant (about sevenfold increase relative to the wild-type strain). In stationary phase, the cAMP level slightly decreased in the wild-type strain, as previously reported [33], and did not change significantly in the *pde1* or the *ira2* mutants although the *ira2* mutant maintained about twofold higher basal level than wild-type cells. Surprisingly, significant increases in the cAMP level were observed in the *pde2* mutant (∼twofold relative

Table 2
Determination of cAMP levels

Strain	cAMP (fmol/10^5 cells)[a]	
	Exponential phase	Stationary phase
CY4 (wt)	3.20 ± 0.07	2.22 ± 0.47
pde1Δ::TRP1	3.24 ± 0.27	3.34 ± 0.06
pde2Δ::HIS3	6.53 ± 0.03	12.5 ± 0.75
pde1Δ::TRP1 pde2Δ::HIS3	22.3 ± 1.18	87.5 ± 4.66
ira2Δ::HIS3	6.81 ± 0.01	5.41 ± 0.33

[a] Cells were grown on YEPD medium. Samples for exponential phase were taken after 6–7 h growth (A_{600} of 1), and samples for stationary phase were taken after 48 h growth. The data shown are means (±SE) from a representative experiment performed in triplicate. Experiments were repeated at least three times with similar results.

to exponential phase) and in the *pde1 pde2* mutant (~fourfold relative to exponential phase). These data indicated that stress sensitivity, in general, correlates with cellular levels of cAMP, and that the basal cAMP level, especially in stationary phase, is mainly affected by cAMP phosphodiesterases.

Depletion of Ras activity can suppress stress sensitivity caused by ira2 deletion, whereas it cannot suppress stress sensitivity caused by lack of cAMP phosphodiesterases

We next investigated whether the Ras–cAMP pathway is essential for *IRA2* or *PDE2*-mediated stress responses. First, we examined whether stress sensitivity of the *ira2* mutant could be nullified by blocking Ras, adenylate cyclase, or PKA, which are the key components of the Ras–cAMP pathway. Freez–thaw resistance of the *ira2* mutant was recovered to wild-type levels upon deletion of *RAS2* (Fig. 3A). Likewise, introduction of a temperature-sensitive mutation of *CYR1*/*CDC35*, encoding adenylate cyclase, rendered the *ira2* mutant freeze–thaw resistant at the restrictive temperature (Fig. 3B). Depletion of PKA activity could also nullify the *ira2* phenotype (Fig. 3C). Introduction of the *tpk1 tpk2* double deletion conferred wild-type levels of freeze–thaw resistance to the *ira2* mutant. Since the expression of *TPK3* is naturally low [42], deletion of both *TPK1* and *TPK2* was expected to significantly reduce PKA activity. Taken together, these data showed that *IRA2* mediates stress responses through the Ras–cAMP pathway.

We next examined whether depletion of Ras activity could suppress the stress sensitivity caused by lack of cAMP phosphodiesterases. If Ras is essential for determining the cAMP level in stationary phase, depletion of Ras activity might neutralize stress sensitivity caused by deletion of *PDE* genes. To test this, a set of haploid segregants carrying *ras2*, *pde1 pde2*, or *ras2 pde1 pde2* were generated from the diploid strain Pde12/ras2(II). Since *RAS1* expression is strongly repressed during respiration [43], the *ras2* deletion was expected to deplete Ras activity in the culture. Furthermore, the data with the *ira2* mutant above also showed that *ras2* deletion alone was sufficient to deplete Ras activity. Nevertheless, deletion of *RAS*2 could not effectively rescue the *pde1 pde2* mutant cells from the stress caused by freeze–thawing or NaCl, although it could suppress heat-shock sensitivity of the mutant (Figs. 4A and B), indicating that Ras is partially required for a complete heat-shock response. In contrast, PKA appeared to be essential for *PDE2*-mediated stress responses. We examined the effect of *PDE2* overexpression in a *bcy1* mutant strain, dbcy1, lacking the regulatory subunits of PKA, and observed that the overexpression could not rescue the *bcy1* mutant although it was sufficient to confer stress tolerance to wild-type cells, *msn2 msn4* mutants and *yap1* mutants (Figs. 5A and B). Taken together, these data indicated that yeast may utilize, in addition to Ras, other signaling proteins to activate PKA in stationary phase, and these may function through the *PDE2* gene product.

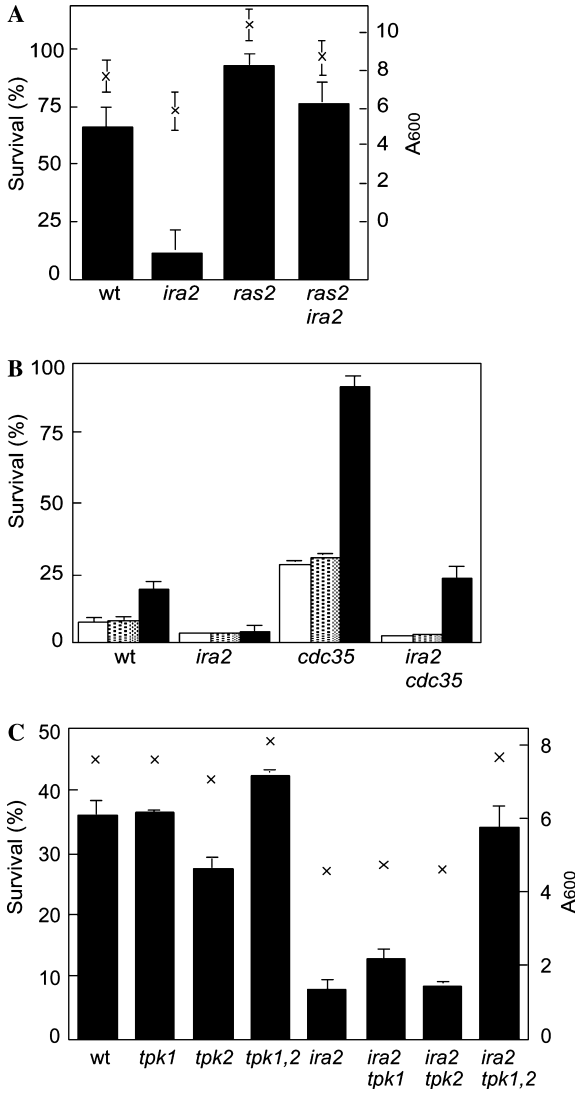

Fig. 3. Stress-sensitivity of the *ira2* mutant can be neutralized by blocking the Ras-adenylate cyclase and protein kinase A pathway. Freeze–thaw tolerance of strains grown on YEPD medium was determined. Percentage survival (column) is expressed relative to the culture viability immediately prior to freezing (%). (×) Indicates A_{600} of each culture at the time points. (A) Strains used were 2 day culture of haploid progeny of the heterozygous diploid, Fsm/ras2(II) (*ira2*/*ras2*). The data shown are means (±SE) for four independent sets of dissection products. (B) One set of tetrad dissection products of the heterozygous diploid Fsm/LRA85 (*ira2*/*cdc35*) was grown to exponential phase (A_{600} of 2) at 22 °C (blank) and shifted to 37 °C (dark) or maintained at 22 °C (dotted) for 1 h prior to freezing. The data shown are means (±SE) from a representative experiment performed in triplicate. (C) Strains used were 2 day culture of haploid progeny of the heterozygous diploid, Fsm/MB12 (*ira2*/*tpk1*/*tpk2*). The data shown are means (±SE) for three independent sets of dissection products. Experiments were repeated at least three times with similar results.

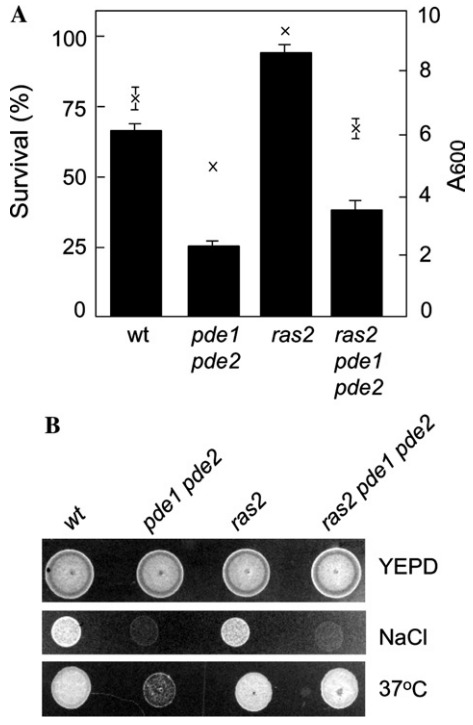

Fig. 4. Stress-sensitivity of the *pde1 pde2* mutant cannot be neutralized by depleting Ras activity. Strains used were dissection products of the heterozygous diploid, Pde12/ras2(II) (*pde1 pde2*/*ras2*), grown on YEPD medium. (A) Freeze–thaw tolerance of 2 day culture was measured. Percentage survival (column) is expressed relative to the culture viability immediately prior to freezing (%). (×) Indicates A_{600} of each culture at the time points. The data shown are means (±SE) for three independent sets of dissection products. (B) The resistance of 1 day cultured cells was determined by spotting 5 μl of each diluted fraction (A_{600} of 0.1) onto YEPD plates containing NaCl or by incubating cells at 37 °C. Experiments were repeated using two other tetrad sets with similar results.

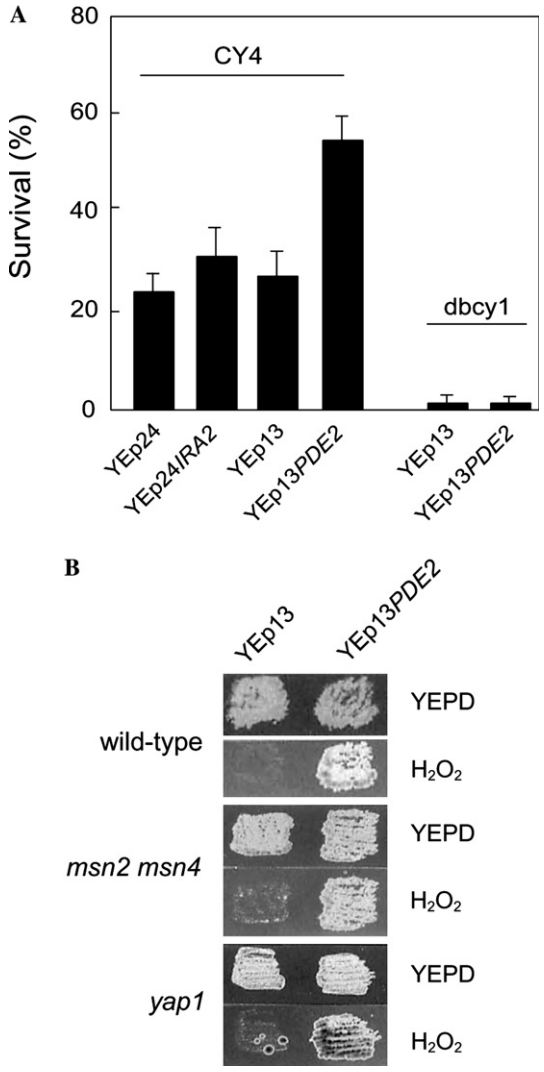

Fig. 5. Overexpression of *PDE2* confers stress tolerance to wild-type strains as well as *yap1* or *msn2 msn4* mutants. (A) Strains CY4 or dbcy1 were transformed with YEp13*PDE2* or YEp24*IRA2*, and freeze–thaw tolerance of exponentially growing cells (A_{600} of 1) was determined. Percentage survival (column) is expressed relative to the culture viability immediately prior to freezing (%). The data shown are means (±SE). p value obtained for *PDE2* overexpression in CY4 was 0.0001 ($n = 10$). (B) Strains W303-1A (wt), Wmsn2msn4 (*msn2 msn4*), and JY29 (*yap1*) transformed with YEp13*PDE2* or the control vector YEp13 were replica plated to YEPD plates containing 3 mM H_2O_2. Experiments were repeated at least three times with similar results.

Discussion

This study shows that control of cAMP level is important for the intrinsic stress tolerance of yeast cells. Comparison of the *ira2* mutants with cells lacking the Yap1 transcription factor, a crucial component in stress defence [38–40], clearly demonstrated that the Ras–cAMP pathway is essential for stress defence. This study also highlights how negative regulators on different branches of the Ras–cAMP pathway are involved in determining the intrinsic stress tolerance and basal cAMP levels.

The role of cAMP is best understood as a second messenger for transient responses to nutrient signals [44,45]. Our study suggests that this second messenger also has a role in determining the cellular stress tolerance. The basal level of cAMP in stationary phase appears to be mainly determined by Pde2p, as demonstrated by mutants deficient in the cAMP phosphodiesterases. *IRA2* did not affect the cAMP level in stationary phase as much as *PDE2* although control of cAMP synthesis by *IRA2* also appeared to be important for determining some intrinsic stress tolerance. *PDE1* appears to be required only when *PDE2* is not present, in other words, when cAMP levels can rise to meet the K_m of the low-affinity enzyme. Therefore, while low-affinity cAMP phosphodiesterase has a specific function in regulating cAMP level in response to signals of glucose or intracellular acidification [28,31], the high-affinity cAMP phosphodiesterase is essential for determining intrinsic stress tolerance of yeast cells.

cAMP phosphodiesterase catalyzes the hydrolysis of cAMP to AMP. AMP is considered to be involved in

stress responses in mammalian systems through the AMP-activated protein kinase [46]. However, in yeast, there is no evidence for AMP-mediated activation of Snf1p, the yeast homologue of the mammalian AMP-activated protein kinase [47,48]. Since overexpression of *PDE2* could not confer stress resistance to mutant cells lacking the regulatory subunits of PKA, cAMP phosphodiesterase in yeast appears to mediate its effect exclusively through PKA inhibition. Thus, yeast may not utilize AMP for stress signaling.

Overexpression of *PDE2* could confer stress resistance to mutants lacking the Yap1 or the Msn2/4 transcription factors, suggesting that PKA can employ diverse stress defence systems with overlapping function. Thus, modulation of PKA activity would be an efficient way to control stress tolerance of yeast. *PDE2* overexpression was more efficient in conferring stress tolerance than *IRA2*. *PDE2* overexpression could even augment stress tolerance of wild-type cells (Fig. 5), but *IRA2* overexpression could rescue only the *ira2* mutant (Figs. 1C and D). This could be due to limited availability of Ras proteins in cells. Therefore, it appears that *PDE2*-mediated cAMP control has greater potential in regulating PKA activity in response to various stresses, and control of *PDE2* activity may be a critical step for determining intrinsic stress resistance of yeast cells.

While the *ira2* mutant showed in general a similar pattern of stress responses as the *pde2* mutant, it displayed relatively high sensitivity to CHP. It was even more sensitive than the *pde1 pde2* double-deletion mutant, which showed higher sensitivity than the *ira2* mutant to other stress conditions. This suggests that *IRA2* may have a specific role in the CHP-induced stress response, and thus supports the notion that each regulatory component of the Ras–cAMP pathway can respond to different stress signals. For example, Ssa1p, a chaperone of the *HSP70* superfamily, has been shown to interact with Cdc25p to reduce PKA activity, indicating that Cdc25p connects heat-shock signaling to the Ras–cAMP pathway [49].

In addition to Ras, yeast cells appear to utilize other signaling proteins to control cAMP levels in stationary phase. Deletion of *RAS2* could not suppress stress sensitivity of the *pde1 pde2* mutant, although *ras2* deletion could suppress *ira2* mutation. This indicates that adenylate cyclase can also be activated by other pathways in stationary phase. It has been shown that the G-protein coupled receptor system, GPR1–GPA2, can activate adenylate cyclase in response to glucose in a Ras-independent manner [50–53]. Similarly, yeast cells may have a mechanism for activation of adenylate cyclase in stationary phase, which is independent of Ras. Thus, it will be important to identify signaling proteins involved in the control of cAMP levels and stress tolerance in stationary phase. Our study suggests that the basal level of cAMP maintained in stationary phase of yeast is a result of a dynamic balance between its rates of synthesis and degradation.

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References

[1] P.K. Herman, Curr. Opin. Microbiol. 5 (2002) 602–607.
[2] T. Belazzi, A. Wagner, R. Wieser, M. Schanz, G. Adam, A. Hartig, H. Ruis, EMBO J. 10 (1991) 585–592.
[3] A. Smith, M.P. Ward, S. Garrett, EMBO J. 17 (1998) 3556–3564.
[4] A. Stanhill, N. Schick, D. Engelberg, Mol. Cell. Biol. 19 (1999) 7529–7538.
[5] H. Garreau, R.N. Hasan, G. Renault, F. Estruch, E. Boy-Marcotte, M. Jacquet, Microbiology 146 (2000) 2113–2120.
[6] R. Hasan, C. Leroy, A.D. Isnard, J. Labarre, E. Boy-Marcotte, M.B. Toledano, Mol. Microbiol. 45 (2002) 233–241.
[7] C. Charizanis, H. Juhnke, B. Krems, K.D. Entian, Mol. Gen. Genet. 262 (1999) 437–447.
[8] D. Hirata, S. Harada, H. Namba, T. Miyakawa, Mol. Gen. Genet. 249 (1995) 257–264.
[9] H. Dihazi, R. Kessler, K. Eschrich, J. Biol. Chem. 279 (2004) 23961–23968.
[10] J.I. Park, C.M. Grant, P.V. Attfield, I.W. Dawes, Appl. Environ. Microbiol. 63 (1997) 3818–3824.
[11] P. Van Dijck, M.F. Gorwa, K. Lemaire, A. Teunissen, M. Versele, S. Colombo, F. Dumortier, P. Ma, A. Tanghe, A. Loiez, J.M. Thevelein, Int. J. Food Microbiol. 55 (2000) 187–192.
[12] T. Toda, I. Uno, T. Ishikawa, S. Powers, T. Kataoka, D. Broek, S. Cameron, J. Broach, K. Matsumoto, M. Wigler, Cell 40 (1985) 27–36.
[13] T. Toda, S. Cameron, P. Sass, M. Zoller, J.D. Scott, B. McMullen, M. Hurwitz, E.G. Krebs, M. Wigler, Mol. Cell. Biol. 7 (1987) 1371–1377.
[14] T. Toda, S. Cameron, P. Sass, M. Zoller, M. Wigler, Cell 50 (1987) 277–287.
[15] D.D. Hall, D.D. Markwardt, F. Parviz, W. Heideman, EMBO J. 17 (1998) 4370–4378.
[16] A. Reinders, N. Burckert, T. Boller, A. Wiemken, C. de Virgilio, Genes Dev. 12 (1998) 2943–2955.
[17] L. Dejean, B. Beauvoit, A.P. Alonso, O. Bunoust, B. Guerin, M. Rigoulet, Biochim. Biophys. Acta 1554 (2002) 159–169.
[18] M. Bolte, P. Dieckhoff, C. Krause, G.H. Braus, S. Irniger, Microbiology 149 (2003) 1205–1216.
[19] H. Dihazi, R. Kessler, K. Eschrich, Biochemistry 42 (2003) 6275–6282.
[20] S.C. Howard, A. Hester, P.K. Herman, Genetics 165 (2003) 1059–1070.
[21] D. Muller, S. Exler, L. Aguilera-Vazquez, E. Guerrero-Martin, M. Reuss, Yeast 20 (2003) 351–367.
[22] K.J. Verstrepen, G. Derdelinckx, J.P. Dufour, J. Winderickx, I.S. Pretorius, J.M. Thevelein, F.R. Delvaux, FEMS Yeast Res. 4 (2003) 285–296.

[23] K. Tanaka, K. Matsumoto, A. Toh-E, Mol. Cell. Biol. 9 (1989) 757–768.
[24] K. Tanaka, M. Nakafuku, T. Satoh, M.S. Marshall, J.B. Gibbs, K. Matsumoto, Y. Kaziro, A. Toh-e, Cell 60 (1990) 803–807.
[25] K. Tanaka, M. Nakafuku, F. Tamanoi, Y. Kaziro, K. Matsum\oto, A. Toh-e, Mol. Cell. Biol. 10 (1990) 4303–4313.
[26] K. Tanaka, B.K. Lin, D.R. Wood, F. Tamanoi, Proc. Natl. Acad. Sci. USA 88 (1991) 468–472.
[27] P. Sass, J. Field, J. Nikawa, T. Toda, M. Wigler, Proc. Natl. Acad. Sci. USA 83 (1986) 9303–9307.
[28] J. Nikawa, P. Sass, M. Wigler, Mol. Cell. Biol. 7 (1987) 3629–3636.
[29] J. Londesborough, K. Suoranta, J. Biol. Chem. 258 (1983) 2966–2972.
[30] K. Suoranta, J. Londesborough, J. Biol. Chem. 259 (1984) 6964–6971.
[31] P. Ma, S. Wera, P. Van Dijck, J.M. Thevelein, Mol. Biol. Cell 10 (1999) 91–104.
[32] J.B. van der Plaat, Biochem. Biophys. Res. Commun. 56 (1974) 580–587.
[33] M. Russell, J. Bradshaw-Rouse, D. Markwardt, W. Heideman, Mol. Biol. Cell 4 (1993) 757–765.
[34] J.F. Cannon, K. Tatchell, Mol. Cell. Biol. 7 (1987) 2653–2663.
[35] C.M. Grant, F.H. Maciver, I.W. Dawes, Mol. Microbiol. 22 (1996) 739–746.
[36] R.D. Gietz, R.H. Schiestl, A.R. Willems, R.A. Woods, Yeast 11 (1995) 355–360.
[37] A. Baudin, O. Ozier-Kalogeropoulos, A. Denouel, F. Lacroute, C. Cullin, Nucleic Acids Res. 21 (1993) 3329–3330.
[38] W.S. Moye-Rowley, K.D. Harshman, C.S. Parker, Genes Dev. 3 (1989) 283–292.
[39] W.M. Toone, B.A. Morgan, N. Jones, Oncogene 20 (2001) 2336–2346.
[40] G. Georgiou, Cell 111 (2002) 607–610.
[41] J.I. Park, C.M. Grant, M.J. Davies, I.W. Dawes, J. Biol. Chem. 273 (1998) 22921–22928.
[42] M.J. Mazon, M.M. Behrens, E. Morgado, F. Portillo, Eur. J. Biochem. 213 (1993) 501–506.
[43] D. Breviario, A. Hinnebusch, J. Cannon, K. Tatchell, R. Dhar, Proc. Natl. Acad. Sci. USA 83 (1986) 4152–4156.
[44] J. Francois, J.L. Parrou, FEMS Microbiol. Rev. 25 (2001) 125–145.
[45] F. Rolland, J. Winderickx, J.M. Thevelein, FEMS Yeast Res. 2 (2002) 183–201.
[46] B.E. Kemp, D. Stapleton, D.J. Campbell, Z.P. Chen, S. Murthy, M. Walter, A. Gupta, J.J. Adams, F. Katsis, B. van Denderen, I.G. Jennings, T. Iseli, B.J. Michell, L.A. Witters, Biochem. Soc. Trans. 31 (2003) 162–168.
[47] P. Sanz, Biochem. Soc. Trans. 31 (2003) 178–181.
[48] A. Woods, M.R. Munday, J. Scott, X. Yang, M. Carlson, D. Carling, J. Biol. Chem. 269 (1994) 19509–19515.
[49] M. Geymonat, L. Wang, H. Garreau, M. Jacquet, Mol. Microbiol. 30 (1998) 855–864.
[50] S. Colombo, P. Ma, L. Cauwenberg, J. Winderickx, M. Crauwels, A. Teunissen, D. Nauwelaers, J.H. de Winde, M.F. Gorwa, D. Colavizza, J.M. Thevelein, EMBO J. 17 (1998) 3326–3341.
[51] Y. Xue, M. Batlle, J.P. Hirsch, EMBO J. 17 (1998) 1996–2007.
[52] L. Kraakman, K. Lemaire, P. Ma, A.W. Teunissen, M.C. Donaton, P. Van Dijck, J. Winderickx, J.H. de Winde, J.M. Thevelein, Mol. Microbiol. 32 (1999) 1002–1012.
[53] M.C. Lorenz, X. Pan, T. Harashima, M.E. Cardenas, Y. Xue, J.P. Hirsch, J. Heitman, Genetics 154 (2000) 609–622.
[54] F. Estruch, M. Carlson, Mol. Cell. Biol. 13 (1993) 3872–3881.