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The Role of the Protein Kinase-A Pathway in the Response to Alkaline pH Stress in Yeast

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ABSTRACT

Exposure of *Saccharomyces cerevisiae* to alkaline pH represents a stress condition that generates a compensatory reaction. Here we examine a possible role of the protein kinase-A (PKA) pathway in this response. The phenotypic analysis reveals that mutations that activate the PKA pathway (*ira1 ira2*, *bcy1*) tend to cause sensitivity to alkaline pH, whereas its deactivation enhances tolerance to this stress. We observe that alkalinisation causes a transient decrease in cAMP, the main regulator of the pathway. Alkaline pH causes rapid nuclear localization of the PKA-regulated Msn2 transcription factor which, together with Msn4, mediates a general stress response by binding to STRE sequences in many promoters. Consequently, a synthetic STRE-LacZ reporter shows a rapid induction in response to alkaline stress. An *msn2 msn4* mutant is sensitive to alkaline pH, and transcriptomic analysis reveals that after 10 minutes of alkaline stress, the expression of many induced genes (47%) depends, at least in part, on the presence of Msn2 and Msn4. Taken together, these results demonstrate that inhibition of the PKA pathway by alkaline pH represents a substantial part of the adaptive response to this kind of stress and that this response involves Msn2/Msn4-mediated genome expression remodelling. However, the relevance of attenuation of PKA in high pH tolerance is likely not restricted to regulation of Msn2 function.

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INTRODUCTION

The regulation of the activity of the cAMP-PKA pathway plays a major role in the control of metabolism and cell proliferation in yeast cells, mostly linked to the available carbon source. In *S. cerevisiae*, for instance, in response to a rapidly fermentable carbon source, such as glucose, the pathway activates the Cyr1 adenylate cyclase, which results in a transient increase in cAMP levels. PKA is a heterotetramer composed of two catalytic subunits and two regulatory subunits. The catalytic subunits can be encoded by three, largely redundant genes (*TPK1*, *TPK2* and *TPK3*), whereas the regulatory subunits are encoded by a single gene (*BCY1*). Binding of the second messenger cAMP to the regulatory subunits, results in dissociation of the complex and activation of PKA. Restoration of cAMP levels is controlled by the low- and high-affinity phosphodiesterases, encoded by *PDE1* and *PDE2*, respectively, which hydrolyze cAMP to AMP. Sequentially, PKA affects diverse downstream targets, often at the gene transcription level, including stimulation of cell growth and cell cycle progression, upregulation of glycolysis, downregulation of gluconeogenesis and mobilization of glycogen and trehalose [1-5].

PKA can be activated in response to glucose by two parallel signalling pathways. The first one involves the Ras1 and Ras2 small GTPases, which are activated by glucose uptake and phosphorylation. The active (GTP-bound) Ras proteins increase the activity of the adenylate cyclase. In turn, the GDP/GTP exchange on the Ras proteins is controlled by the guanine nucleotide exchange factors (GEF) Cdc25 and Sdc25. The reverse process is accelerated by the Ira proteins (encoded by *IRA1* and *IRA2*), which act as Ras GTPase activating proteins (GAP) and maintain Ras in the GDP-bound, inactive state. The second involves Gpr1, a putative G protein-coupled receptor, and its Gα protein Gpa2. Both pathways converge to activate adenylate cyclase, resulting in the generation of cAMP [1-3].

Activation of PKA has a major impact on gene expression. Consequently, several transcription factors are among the known PKA targets. Two of those are Msn2 and Msn4, which mediate the transcription of the so-called stress response element (STRE)-controlled genes [6-8]. STRE-regulated genes are involved in important processes, such as carbohydrate metabolism and growth regulation, as well as in adaption to diverse types of stress, including heat, DNA damage, oxidative and osmotic stresses [9-12]. Under growth–promoting conditions (growth on glucose, absence of stress,..) Msn2 and Msn4 are phosphorylated and reside in the cytosol. Upon glucose exhaustion or other stress conditions [13], they become hypophosphorylated and translocate to the nucleus, where they induce expression of the STRE-controlled genes. PKA plays a very important role inhibiting nuclear import of Msn2/4, either through direct phosphorylation of their nuclear localization signal [13-15] or indirectly via the protein kinases Yak1 and Rim15.

S. cerevisiae grows far better at acidic than at neutral or alkaline pH and, consequently, even a modest alkalinization of the medium represents a stress situation that is able to trigger a compensatory multifactorial response (see [16] for review). Alkaline stress activates diverse signaling pathways, including the Rim101-Nrg1 [17;18], the calcium / calcineurin [19-21], and the Wsc1-Pkc1-Slt2 MAP kinase pathways [22]. Alkalinisation of the environment also disturbs nutrient homeostasis, as deduced from its impact on iron/copper and phosphate uptake/utilization pathways [19;23]. Work in our laboratory in the past years has evidenced that alkaline pH stress has also a profound impact on the expression of genes encoding glucose uptake and metabolism-related proteins, in the sense that exposure to high pH would mimic a situation of glucose starvation [20;21]. Given the strong link between carbohydrate metabolism and the PKA pathway, we speculated that alkaline stress might involve changes in the activity of this pathway and, specifically, that the correct adaptation to high pH could entail its downregulation. Indeed, in this paper we show that alkaline pH stress causes a transient decrease in cAMP levels and that change in the activity of the PKA pathway alters tolerance to alkaline pH. Our data also indicates that the adaptive response to high pH involves PKA-regulated, Msn2/Msn4-mediated gene remodelling.

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Experimental

Yeast strains and growth conditions

Yeast cells (strains are listed in Table 1) were grown at 28°C in YPD medium (10 g/l yeast extract, 20 g/l peptone, and 20 g/l dextrose) or, when carrying plasmids, in synthetic complete medium [24] containing 2% glucose and lacking the appropriate selection requirements. Single *kanMX* deletion mutants, in the BY4741 background, except MAR231, were generated in the context of the *Saccharomyces* Genome Deletion Project [25].

Strain MAR231 was made by transforming BY4741 with a *bcy1::kanMX4* cassette obtained from the strain KKY385 [26] by PCR amplification using primers 5´-bcy1_disr (GAGGAGCATACGACTTCGGC) and 3´-bcy1_disr (CTGTCTTGTAGATCCTTTGG). Strains CCV35 and CCV36 were constructed as follows. A 1.6 Kbp *pde1::kanMX4* cassette was amplified from genomic DNA of the BY4741 *pde1::kanMX4* strain with oligonucleotides 5'-pde1 (CAAGGATCGTTACCCGGTA) and 3'-pde1 (GACTTATGTTGGGATAGGGG). The purified DNA fragment was used to transform strains W303 or PM942 [27], to yield CCV35 and CCV36, respectively. Strains CCV37 and CCV38 were obtained transforming W303-1A wild type and MCY5278 strains with a *SNF1::LEU2* disruption cassette [21] and strains CCV174 and CCV175 were generated by transforming W303-1A and MCY5278 strains with the 2.1 kb *nrg1::nat1* cassette from plasmid pBS-nrg1::nat1 as described in [28]. Strain AGS66 contains an integrated STRE(7x)-*lacZ* reporter system at the *URA3* locus [29].

Plasmids

The following plasmid constructs were used in this work. The reporter plasmid p*HXK1*-lacZ was generated as follows. The *HXK1* upstream DNA region containing -624 and +39, relative to the starting ATG, was amplified by PCR with added BamHI/PstI restriction sites and cloned into the same sites of YEp357 [30]. Plasmid YCp50-RAS2^{Ala18Val19} [31] expresses the *RAS2*^{Ala18Val19} hyperactive Ras2 allele [32] from the centromeric YCp50. Plasmid pG2CT-112.2 expresses the constitutively active *GPA2*^{R273A} allele from the episomal YEplac112 backbone [31]. Plasmid pAMS366 contains a tandem of four CDRE elements from the FKS2 gene fused to the lacZ reporter [33]. pPHO84-LacZ contains a PHO84-LacZ reporter fusion as described in [19]. pKC201 contains the *ENA1* promoter fused to *LacZ* ([34;35].

Growth Tests

The sensitivity of different yeast strains to alkaline pH was assayed by drop test on YPD plates containing 50 mM TAPS adjusted with KOH at different pH values. Growth in liquid medium at high pH was performed in 5 ml cultures or in 96-well plates (250 µl), at 28 °C in YPD medium buffered with 50 mM TAPS and adjusted with KOH at the indicated pH values. Growth was monitored by measuring the *A* at 660 nm. BY4742 and DC90 cells, at an initial A_{660} of 0.001, were grown for 17 and 24 h respectively. Strains prepared from the W303-1A genetic background were grown from an initial A_{660} of 0.01 for 17 h (wild type and MCY5278 strains) and for 21h (CCV37 and CCV38 strains).

Measurements of cAMP levels

Measurements were made as described in [36]. Briefly, BY4741 cells were grown to an OD_{660} of approximately 1.0 in YPD at 28ºC, and then the culture was centrifuged and resuspended in YPD containing 50 mM TAPS pH 8. Aliquots of 10 ml were filtered at the indicated times and extracted in the cold with 1 ml 2 M perchloric acid. After neutralization with 1 ml of 1.8 M KOH and 0.4 M $KHCO_3$ and centrifugation, samples were purified with Amprep SAX minicolumns (Amersham, code RPN 1918), eluted with methanol-HCl and dried under vacuum. Assays were performed by a competitive binding method with the Amersham cAMP enzymeimmunoassay (EIA) system (code RPN 225). For calculation of yeast concentrations, one unit of absorbance at 660 nm was equivalent to 2.6 mg wet weight/ml. In other set of experiments, pH was raised to 8.2 by addition of KOH (35 mM, final concentration). Control cells received the same concentration of KCl.

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β-Galactosidase Activity Assay

Yeast cells were grown to saturation in the appropriate dropout media and then inoculated into YPD (or YP plus 4% glucose, when indicated) at pH 5.5. Growth was resumed until A_{660} 0.5–0.7, and cultures were centrifuged for 5 min at 1620 × *g*. Cells were resuspended in YPD or YP 4% glucose where indicated (no induction) or YPD (or YPD 4% glucose) plus 50 mM TAPS adjusted to pH 8.0 (alkaline stress), and growth was resumed for the indicated times. In all cases, β-galactosidase activity was measured as described previously [37].

Microscopy techniques

For Msn2 subcellular localization experiments, the indicated strains were transformed with plasmid pMSN2-GFP (a generous gift of F. Estruch, University of Valencia, Spain), a YCplac111-based vector that contains a C-terminal Msn2-green fluorescent protein fusion [13]. Cells were grown in YPD or YP 2% ethanol until an A_{660} of 0.8 to 1.0 was reached. Cultures (5 ml) were treated as follows: addition of 100 µl of 1 M KCl (control cells, pH 5.5) or addition of 100 µl of 1 M KOH (alkaline stress, pH 8.0). Samples (500 µl) were taken at the appropriate times and fixed for 5 min by adding 30 µl of 37% formaldehyde. Cells were harvested, washed three times with a phosphate-buffered saline solution (PBS), and concentrated 10-fold before visualization. In all cases the cells were visualized with a fluorescein filter using a Nikon Eclipse E800 fluorescence microscope (magnification, x1000). Digital images were captured with an ORCA-ER 4742-80 camera (Hamamatsu) using the Wasabi software. Intracellular distribution of Msn2-GFP was quantified by scoring at least in 200 cells per sample into one of three possible categories: cytoplasmic (fluorescence in the cytoplasm only), nuclear-cytoplasmic (fluorescence in the cytoplasm and nucleus), and nuclear (fluorescence in the nucleus only).

RNA purification, cDNA synthesis, and DNA microarray experiments

For RNA purification, 50 ml of yeast culture (strains W303-1A and MCY5278) was grown at 28°C in YPD medium until an A_{660} of 0.6 to 0.8, KOH or KCl was added from a concentrated stock solution (1 M) to reach a final concentration of 20 mM (pH 8.05 and pH 5.5 respectively). Yeast cells were harvested by filtration after 10 and 30 min and washed with cold water; dried cells were kept at –80 °C until RNA purification. Total RNA was purified using a RiboPure-Yeast kit (Ambion, Inc.) following the manufacturer's instructions. RNA quality was assessed by denaturing 0.8% agarose gel electrophoresis, and RNA quantification was performed by measuring absorbance at 260 nm in a BioPhotometer (Eppendorf). Transcriptional analyses were performed using DNA microarrays containing PCR-amplified fragments from 6014 *S. cerevisiae* open reading frames [20;38]. Fluorescent Cy3- and Cy5-labeled cDNA probes were prepared from 8 µg of purified total RNA by the indirect dUTP labelling method using a CyScribe post-labelling kit (Amersham Biosciences).

Pre-hybridization, hybridization, and washes were carried out as recommended by The Institute for Genomic Research with minor modifications. Briefly, prehybridizations of the DNA microarrays were carried out at 42 °C for 1 h in a solution containing 5× SSC, 0.1% SDS and 1% bovine serum albumin. For hybridization, dried Cy3- and Cy5-labeled probes were resuspended in 35 µl of hybridization solution (50% formamide, 5× SSC, 0.1% SDS) each and mixed. Five µg of salmon sperm DNA was added to the mix before denaturation for 3 min at 95 °C. DNA microarrays were hybridized in an ArrayBooster hybridization station (Sunergia Group) for 14 h at 42 °C. The scanner ScanArray 4000 (Packard Instrument Co.) was used to obtain the Cy3 and Cy5 images with a resolution of 10 µm. The fluorescent intensity of the spots was measured and processed using the GenePix Pro 6.0 software (Molecular Devices). Spots with either diameter smaller than 120 µm, or fluorescence intensity for Cy3 and Cy5 lower than 150 units were not considered for further analysis.

For each condition assayed, two independent experiments were performed, and dye swapping was carried out for each experiment. Microarray data was deposited at the Gene Expression Omnibus (GEO) and can be retrieved under accession number GSE27925. Data from different experiments were combined, and the mean was calculated. A given gene was considered to be induced or repressed when the mean of the ratios (alkaline stress *versus* no stress) was >2.0 or <0.50, respectively. Software from the GEPAS server (gepas.bioinfo.cipf.es/) was used to carry out clustering and other data analyses [39]. According to the expression of these genes in the *msn2 msn4* strain, different levels of

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dependence on these transcription factors were defined. Thus, genes showing a mutant/wild type ratio 0.67 > X > 0.50 were considered "weakly dependent" (WD); those with a ratio 0.50 > X > 0.25 were ranked as "strongly dependent" (SD) and those with a ratio ≤ 0.25 were defined as "totally dependent" (TD). Likewise, genes induced more than 2.5-fold in wild type cells and considered not induced (i.e ratio alkaline stress/no stress) <1.3) in *msn2 msn4* cells were also considered as totally dependent. This score is the same employed in our previous report on calcineurin dependence of high pH response [20].

RESULTS

Manipulation of upstream elements of the PKA pathway affects growth at alkaline pH.

Our starting hypothesis was that adaptation to high pH stress should entail a decrease in the PKA pathway activity. Therefore, we speculated that mutations increasing the activity of the pathway should result in decreased tolerance to alkaline pH. We first tested strains lacking components of the Gpr1/Gpa2 sensing pathway, such as *gpr1*, *gpa2*, *rgs2* (lacking a GAP of Gpa2), *gpb1* or *gpb2* (lacking multistep regulator of cAMP-PKA signalling) (Figure 1A). However, none of these strains showed altered tolerance to alkaline pH (not shown). In contrast, deletion of *ira1 or ira2*, the GAPs of Ras1 and Ras2 results in increased sensitivity. This phenotype was enhanced in the double *ira1 ira2* mutant (Figure 1B). Conversely, expression of the Cdc25 WΔN1 allele, which lacks the N-terminal domain of Cdc25 and elicits constitutive activation of Ras proteins, also results in poor growth at high pH (Figure 1B). All these manipulations lead to increased Ras activity and hyperactivation of the PKA pathway. In contrast, the effect on high pH tolerance of the expression of the Cdc25 WΔN2 allele, which does not lead to increase in PKA activity, was barely noticeable. These results were confirmed by expression of the hyperactive Ras allele $RAS2^{Ala18Val19}$ from a centromeric plasmid [31]. Among other phenotypes, expression of this allele decreases heat shock tolerance. As it can be observed (Figure 1C), expression of the $RAS2^{Ala18Val19}$ allele decreases alkaline pH tolerance. In contrast, expression of a constitutively active form of Gpa2 (pG2CT-112.2) constructed by replacing the arginine at position 273 with an alanine [31] did not alter high pH tolerance.

Alkaline pH stress transiently decreases the levels of cAMP.

The levels of cAMP determine the interaction between the regulatory subunit (Bcy1) and the catalytic subunits (Tpk) of PKA and are, therefore, crucial for the activity of the kinase. We have measured the levels of cAMP after shifting the cells from pH 5.5 to media buffered to pH 8.0 and found that this treatment drastically decreases the concentration of the second messenger in the first 5-15 minutes, followed by a recovery to the initial levels after 30 min of stress (Figure 2A). A similar result was obtained when KOH was directly added to the exponentially growing cultures to raise the pH. In this case, decrease in cAMP levels was apparent even only 2 min after stress (not shown). Raising pH of the liquid medium up to values of 8.2 did not result in detectable cell lysis, as determined by microscopic examination, viability counting, or release of alkaline phosphatase activity to the medium.

Our data was consistent with the possibility that a drop in cAMP levels, leading to downregulation of PKA activity, could be an adaptive strategy to confront high pH stress. Therefore, we considered that mutations directly resulting in increased cAMP levels should be deleterious for cells subjected to alkaline pH stress. The *PDE1* and *PDE2* genes encode the yeast cyclic AMP phosphodiesterase activity, which degrades cAMP to AMP. When the single mutants are grown on alkaline pH plates, a growth defect can be observed for the *pde2* mutant (lacking the high affinity isoform) that is exacerbated in the double *pde1 pde2* mutant strain (Figure 2B).

Lack of Bcy1, the regulatory subunit of PKA, leads to constitutive PKA activity, which results in a variety of phenotypes (impaired growth on different carbon sources, temperature sensitivity, etc., see [2] and references therein). Although the quantification of the effect is difficult, because of the relatively poor growth of the *bcy1* mutant even under standard conditions, we can show that this strain exhibits a dramatic alkaline pH-sensitive phenotype (Figure 2C). The same effect was observed in the W303-1A background (data not shown). Similarly, we tested if a decrease in PKA activity could improve growth at high pH. Since complete lack of PKA activity is not compatible with survival, for

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this experiment we resorted to a strain lacking *TPK1* and *TPK3* and carrying an attenuated allele of *TPK2* (*tpk2*^{w3}) as sole source of PKA activity. As it can be observed in Figure 2D, the relative growth of this strain in alkaline pH medium versus non-restrictive conditions is higher than the wild-type strain. In essence, the data presented so far shows that yeast cells respond to the alkalinisation of the medium with a sharp decrease in the intracellular level of cAMP. The observation that when PKA activity is attenuated cells grow better at high pH, whereas any situation resulting in increased PKA activity makes cells more sensitive to this condition, supports the notion that downregulating PKA activity contributes to adaptation to alkaline pH stress.

Alkaline pH stress promotes entry of Msn2 in the nucleus and STRE-dependent promoter activation.

Nuclear localization of Msn2, a transcription factor that, together with Msn4, regulates the general stress response of *S. cerevisiae*, is controlled by PKA activity [13;14;40]. We considered that alkaline pH-triggered downregulation of the PKA activity could result in the translocation of Msn2 to the nucleus and that this transition might have a relevant effect in the pH-induced adaptive response. To test this possibility, an Msn2-GFP fusion was introduced into wild type cells and the cultures subjected to alkaline treatment. As it can be observed in Figure 3A and B, exposure to high pH (8.0) triggers an almost immediate entry of Msn2 into the nucleus, which reached a maximum after 5 min (Figure 3B). Nuclear localization of Msn2 was transient and after 10-15 min the transcription factor was again mostly cytosolic. Interestingly, when the same experiment was carried out in a strain lacking the *IRA2* gene (sensitive to alkaline pH, see Figure 1B), entry of Msn2 to the nucleus was somewhat delayed and nuclear localization was a less general effect than in the wild type strain. Nuclear translocation of Msn2 was also evaluated in cells subjected to alkaline stress (pH 8.0) growing in the presence of ethanol as carbon source (Figure 3B). In this case, Msn2 showed both cytoplasmic and nuclear/cytoplasmic distribution in unstressed cells. Interestingly, exposure of cells to alkalinisation resulted in only a modest increase in the percentage of cells showing nuclear/cytoplasmic distribution, but very few cells with only nuclear Msn2. Therefore, subcellular distribution of Msn2 in response to alkaline stress is affected by the carbon source in which cells are grown.

We then investigated if the Msn2 and Msn4 transcription factors are necessary for normal tolerance to high pH stress. As shown in Figure 4A, lack of the transcription factors reduces somewhat tolerance to high pH, suggesting that they are components of the adaptive response to alkalinisation. Remarkably, additional deletion of all three PKA catalytic subunits (which is feasible in the absence of the transcription factors) yields cells that are even more sensitive than the *msn2 msn4* mutant. It should be noted that in this case the *msn2 msn4* strain was transformed with a centromeric plasmid which contains a *TRP1* marker. The introduction of this marker in the reference strain was necessary for accurate comparison because the *tpk msn2,4* strain was constructed using the *TRP1* gene as marker, and it has been reported [41] that the absence of this gene somewhat decreases tolerance to high pH. This results suggested that, besides its role on Msn2,4 function, regulation of PKA activity may exert additional effects on high pH tolerance. This notion was reinforced by the result of the experiment shown in figure 4B. In this case, we observed that expression of the hyperactive form of *RAS2* further decreases high pH tolerance even in the absence of Msn2/Msn4 transcription factors, suggesting again that not all the effects relevant for alkaline tolerance mediated by PKA are necessarily based in the activation of Msn2/Msn4. On the other hand, cells lacking the Snf1 kinase are sensitive to alkaline pH and Snf1 has been reported to repress Msn2 function. Therefore, we considered whether the sensitive phenotype of *snf1* cells could be due to deregulation of Msn2 function. As shown in Figure 4C, *snf1* cells are more sensitive than the *msn2 msn4* mutant to alkaline pH. In addition, deletion of *msn2 msn4* further increases sensitivity to alkaline pH of the *snf1* mutant. Therefore, deregulation of Msn2/Msn4 is not at the basis of the *snf1* alkali-sensitive phenotype.

Because Msn2 is known to bind to STRE elements in response to glucose shortage and other forms of stress, we tested the activation of a synthetic promoter containing a tandem of seven STRE elements fused to the *LacZ* reporter (strain AGS66). As shown in Figure 5A, this promoter exhibited a very fast activation, with a peak of activity after 15 min of shifting cells to pH 8.0. This activation was much faster than that observed when the *LacZ* gene is expressed from a *PHO84* promoter, which is considered a gene with a relatively late response to high pH stress [19] and it was completely abolished in a *msn2 msn4* strain (not shown). Remarkably, the response was also faster than that

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obtained from a synthetic promoter based in a cluster of four copies of the CDRE motif from the calcineurin-responsive *FKS2* gene. It must be noted that calcineurin/Crz1 mediated response is considered to be an early response to alkaline pH stress [19;20]. We also tested the response to alkaline pH of *HXK1*, encoding hexokinase, a gene with predicted STRE elements in its promoter [10] and known to be induced by high temperature (37ºC) and oxidative stress in a *msn2 msn4* dependent way [12]. As it is shown in Figure 5B, expression from the *HXK1* promoter induced by high pH stress was as fast as the one induced by exposure to 37ºC, although the level of β-galactosidase activity declines faster (likely due to yeast-promoted acidification of the medium). Remarkably, in the *msn2 msn4* strain the basal activity of the promoter, as well as the response to both stress conditions, is dramatically reduced. However, a small increase can still be detected after 30 min of exposure to high pH stress, suggesting an Msn2/4-independent input to the promoter, which could be attributed to activation of calcineurin, as this gene was demonstrated to be induced by calcium as well as by high pH [21].

Relevance of Msn2/Msn4 transcription factors in the transcriptional response to high pH stress.

Since these results indicated that Msn2/4 could be responsible for the response of certain genes to alkaline pH stress, we considered necessary to evaluate up to what extent this pathway is responsible for the remodelling of gene expression observed upon exposure to high pH. To this end, DNA microarray analysis was conducted using wild type and *msn2 msn4* strains subjected to pH 8.0 for 10 and 30 min. After 10 min of exposure to high pH, 331 genes were induced at least 2-fold in wild type cells, whereas only 186 were found in the *msn2 msn4* cells strain (from a total number of 3463 genes with valid data, Figure 6 and Supplemental Data).

Gene Ontology (GO) analysis of the genes induced in the wild type strain yielded, as expected, an excess of genes related to carbohydrate metabolism (p<7.74E-09), in particular to trehalose (p<5.05E-08) and glucose (p<1.42E-06) and glycogen (p<1.61E-03) metabolisms. Analysis of the *msn2 msn4* dependence for induction revealed that 157 genes required the presence of the transcription factors for full induction (Figure 6). While dependence was limited in some cases (62 genes were rated as weakly dependent, WD), the majority of the short-term induced genes were strongly (57) or totally (38) dependent of the presence of Msn2 and Msn4. Gene Ontology analysis of the 95 genes defined as SD or TD revealed a strong excess of genes encoding proteins involved in trehalose (p<9.40E-09) and glycogen (p<1.46E-07) metabolism. A similar analysis of genes showing weak dependence provided no distinctive profile.

Exposure of cells to pH 8.0 for 30 min resulted in 241 genes induced in the wild type strain, whereas 296 genes increased expression at least 2-fold in the *msn2 msn4* mutant (Figure 6). In this case, the level of dependence of the transcription factors was much lower (only 50 genes). In addition, the vast majority (40) was weakly dependent; nine were ranked as SD and only one as TD. Gene Ontology analysis of the set of SD plus TD genes did not produce any specific profile. Therefore, Msn2 and Msn4 transcription factors are responsible for the induction of a substantial subset of the early alkali-responsive genes. Analysis of repressed genes showed 146 and 279 genes repressed at least 0.5-fold in the wild type strain after 10 and 30 min of shift to high pH, respectively. The absence of *msn2 msn4* did not result in activation of any of these genes.

The *ENA1* gene encodes a Na^+-ATPase that has been repeatedly reported to be strongly induced upon alkaline pH stimulation. Interestingly, our microarray data indicate that deletion of *MSN2/4* does not affect induction of the ATPase gene at short-term (10 min) but results in higher-than-normal expression after 30 min of stress (Figure 7A). In an attempt to identify a possible cause for this behaviour we searched our microarray raw data for changes in expression of genes encoding known regulatory components of *ENA1*. We observed that after 10 min after alkalinisation of the medium, the expression of *NRG1*, a known repressor of *ENA1* expression, was essentially identical in wild type and *msn2 msn4* cells. However, after 30 min the expression of *NRG1* was decreased by 37% in the *msn2 msn4* strain compared to the wild type. To explore a possible influence of this change on the response of *ENA1*, we evaluated the expression from the ATPase gene promoter in wild type and *msn2 msn4* cells deleted for *NRG1* by means of a *LacZ* reporter. As it can be observed (Figure 7B), high-pH dependent expression from the *ENA1* promoter is stronger in the *msn2 msn4* mutant than in the wild type strain, thus confirming the microarray data. Moreover, the induction observed upon alkaline pH stimulation in the *nrg1* strain is virtually identical to that observed in the *msn2 msn4 nrg1*

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triple mutant, suggesting that changes in the Nrg1 levels could be at the basis of the abnormal response of *ENA1* in the *msn2 msn4* mutant.

DISCUSSION

Exposure to alkaline stress triggers a set of responses that are known to be mediated by different signaling pathways, such as the calcineurin/Crz1, the Rim101, and the Slt2 pathways [16]. PKA mediates a major signaling pathway that is crucial for linking stress responses and cell proliferation. Therefore, the major goal of this work was to evaluate the relevance of the PKA pathway in the adaptive response to high pH stress. We observe that mutations leading to activation of PKA result in decreased alkaline pH tolerance, whereas those that lead to inhibition of the pathway yield cells with increased tolerance. Our results also show that exposure to high pH provokes a sharp and transient decrease in the cAMP levels, which may lead to downregulation of PKA activity. In this regard, a previous work reported a transitory increase of cAMP levels as a result of sudden acidification of the cytosol [42]. It is worth noting that these authors noticed that Gpa2 was not required for the stimulation of cAMP accumulation in response to intracellular acidification. Remarkably, we observe that only alterations in the small G proteins Ras1 and Ras2 branch of the pathway do result in changes in tolerance to alkaline pH, whereas mutations in diverse genes involved in the Gpr1/Gpa2 glucose sensor system do not produce phenotypic effects. This suggests that the observed changes are due to alteration of intracellular glucose metabolism and not to defects in the extracellular glucose detection system and reinforce the notion that changes in cytosolic pH may result in fluctuations in cAMP levels and consequent regulation of PKA activity. In the case of sudden alkalinisation of the medium, the sharp decrease in cAMP levels would inhibit PKA activity and allow cells for adaptation to the stress.

Our results show that alkalinisation of the medium triggers a very fast entry of the Msn2 transcription factor to the nucleus (2-5 min). This time frame is very similar to that observed when cells are shifted to low glucose [13;14]. Interestingly, the fact that we observe only a very modest response in ethanol-grown cells suggests that nuclear transition of Msn2 is related to alkaline stress-induced alterations in glucose signalling. We also show that Msn2 and Msn4 are important for mediating a substantial part of the transcriptional response induced by alkalinisation of the medium. It is conceivable that this response constitutes one of the factors that allow normal tolerance to the stress, since the *msn2 msn4* mutant is sensitive to high pH (Figure 4A). Moreover, our results suggest that regulation of Msn2 function in response to high pH is the result of inhibition of PKA. It is known that intracellular localization of Msn2 can be regulated not only by PKA but also by the Snf1 kinase [43;44] and the TOR pathway [44;45], thus raising the possibility that these two pathways could contribute to the alkaline pH-triggered entry of Msn2 into the nucleus. In fact, activation of the Snf1 pathway has been previously linked to high pH stress [21;28;46]. However, we observe that mutation of *SNF1* yields cells even more sensitive to alkaline pH than those lacking *msn2 msn4*, and that deletion of both factors in a *snf1* background further decreases tolerance. Furthermore, we have observed that, within the time-span shown in Figure 3A, entry into the nucleus of Msn2 is not affected by the lack of the protein kinase Snf1 after alkaline treatment (data not shown). Finally, comparison of the dependence level for Msn2,4 of the short-term transcriptional response to high pH described in this work with the dependence on Snf1, evaluated in a parallel project (Casamayor, A., Ruiz, A., Serrano, R., Platara, M., Ferrer-Dalmau, J. & Ariño, J. unpublished work), indicates low overlap (only 34 of the 157 genes reported as Msn2,4-dependent in this report are found to exhibit some degree of dependence for the Snf1 kinase). As an example, most of the genes encoding enzymes involved in trehalose metabolism whose expression is increased by alkalinisation are Msn2,4-dependent, but not Snf1-dependent. Therefore, our results do not support the notion that activation of Msn2/4 by high pH is mediated by the Snf1 kinase.

The PKA and TOR pathways illustrate diverse ways of functional interaction in the regulation of cell growth, showing some functional overlap [47]. However, a role of the TOR pathway in regulation of Msn2 function in response to high pH is questionable, because with the only exception of the *ure2* strain (a very pleiotropic strain), mutants in genes of the pathway do not show altered tolerance to high pH nor alkalinisation triggers increased expression of well-established readouts for inhibition of the TOR pathway, such as *GAP1*, *GLN1* or *GDH1* (our own data, not shown). Two protein kinases, Yak1 and Rim15, have been reported to modulate Msn2 function in response to PKA

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activation (see [5] for review). However, *yak1* and *rim15* mutant strains are not sensitive to high pH (data not shown) and, furthermore, it has been proposed that Yak1 regulates Msn2 function by an unknown mechanism that does not implicate the control of Msn2 subcellular localization. Therefore, the most likely scenario is that Msn2 entry into the nucleus after high pH stress is caused by inhibition of PKA activity.

Comparison of the time sequence of the events described in our work is rather coherent, with a very fast decrease in cAMP levels and subsequent entry of Msn2 in the nucleus, followed by a massive, fast (10 min), and transient activation of Msn2/4 dependent genes, as deduced by DNA microarray transcriptomic analysis. It is remarkable that, after 30 min of high pH stress, the transcriptional response becomes largely *msn2 msn4*-independent. In fact, our data (Figure 5A) suggest that Msn2/Msn4 may be responsible for the first chronological set of transcriptional responses to alkaline stress, even a faster response than that mediated by the calcineurin/Crz1 pathway, which were currently defined as early responses [19;20].

It has been widely documented that expression of the *ENA1* Na^+-ATPase is potently induced by alkaline pH (see [48] and references therein). An interesting observation derived from our microarray data is that the increase in *ENA1* expression is virtually identical in wild type and *msn2 msn4* cells at short times (10 min), but is further enhanced by the lack of Msn2/4 at 30 min. Interestingly, we observed long time ago a similar effect by using a *LacZ* translational fusion of the *ENA1* promoter and a different *msn2 msn4* mutant strain [19], although at that moment it was not further characterized. Our observations suggest that lack of Msn2/4 results in either activation of a positive regulatory element for *ENA1* expression or removal of a negative regulator. It is well known that Nrg1 acts as a repressor of *ENA1* expression by directly binding to the gene promoter, thus participating in alkaline pH signalling [18;49;50]. We observe here that *NRG1* levels decrease after 30 min of high pH stress and that alkaline pH induction of *ENA1* is not further increased by mutation of *msn2 msn4* in a *nrg1* deletion background. This suggests that the enhanced expression of the ATPase gene in the *msn2 msn4* mutant can be due to a decrease in the amount of the Nrg1 repressor. In this regard, it must be noted that the remarkable effect of lack of *RIM101* in the expression of the ATPase gene [18;19;28], is fully mediated by a rather small change (2.8-fold) in *NRG1* mRNA levels [18].

Our data also suggest involvement of the PKA pathway in high pH tolerance in way that would be independent of the Msn2/4 transcription factors. This is based in the combination of *tpk* and *msn2,4* mutations and in the observation that hyperactivation of *RAS2* in the *msn2 msn4* mutant still increases high pH sensitivity (Figure 4B). A possible explanation could be based in a cooperative role of PKA and calcineurin pathways in the adaptive response to high pH stress. In this regard, it was reported that PKA can phosphorylate Crz1, promoting its exit from the nucleus and thus opposing the action of calcineurin [26]. Therefore, inhibition of PKA may result in further enhancement of calcineurin/Crz1-mediated responses. A further step of complexity may exist in the interaction between the calcineurin and the PKA pathways, as it was recently reported that Crz1 may exert a destabilization effect on Msn2 levels upon calcium treatment [51]. In this context, it is conceivable that entry of Crz1 in the nucleus, following activation of calcineurin by high pH may serve, in addition to activate calcineurin/Crz1-responsive promoters, as a negative feed-back system to avoid persistent activation of Msn2/4-responsive genes. This regulatory system may contribute to the transient Msn2/4-dependent transcriptional effect observed in our work.

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Legends

Figure 1. Effect of mutations in the upstream components of the PKA pathway on alkaline pH tolerance. A) A simplified schematic depiction of the PKA signaling pathway. See Introduction for additional information. B) *Upper panel*. Three dilutions of cultures of wild type BY4741 cells and the isogenic *kanMX* disruption derivatives *ras1, ras2*, *sds25* and *ira2* mutants were spotted on YPD plates adjusted at the indicated pHs. *Middle panel*. Wild type strains W303-1A and its *ira1::LEU2* (strain SC7), *ira2::URA3* (SC8) *and ira1::LEU2 ira2::URA3* (PM903) derivatives were spotted as indicated above. *Lower panel*. Wild type strains W303-1A and isogenic cells expressing the WΔN1 or WΔN2 alleles of *CDC25* were spotted. All plates were incubated for 3 days. C) Wild type strain W303-1A was transformed with the indicated plasmids. Positive clones grown overnight in synthetic selective medium and spotted on YPD plates adjusted at the indicated pH and growth was monitored after 2 days. YCp50-*RAS2** expresses a hyperactive allele ($Ras2^{Ala18Val19}$) of the Ras protein. YEp-*GPA2** generates a constitutively active $Gpa2^{R273A}$ version of the protein.

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Figure 2. Changes in PKA pathway activity influence high pH tolerance. A) Wild type (WT) BY4741 cells were subjected to alkaline pH stress as described in Materials & Methods and cultures processed for cAMP determination. Data correspond to the means ± S.E.M of at least four independent experiments. B) Wild type W303-1A cells and its derivatives *pde1* (strain CCV35), *pde2* (PM942) and *pde1 pde2* (CCV36) were spotted on YPD plates adjusted at the indicated pHs. Growth was monitored after 2 days. C) Strain BY4741 (WT) and its *bcy1* derivative (strain MAR231) were grown on YPD plates adjusted at the indicated pH for 3 days. D) Wild type strain BY4742 (open bars) and its derivative DC90 (*tpk1 tpk2*^{w3} *tpk3*, closed bars) were grown on liquid YPD media as described in Materials and Methods. Growth is represented as percentage over the same strain at initial pH 5.5. Data are the average ± SEM from two independent experiments.

Figure 3. Alkaline pH triggers nuclear entry of the Msn2 transcription factor. Cultures of wild type BY4741 (WT) and its *ira2::kanMX* derivative (right panels) were transformed with plasmid pMsn2-GFP and subjected to pH stress (8.0) on YPD medium as described in the text. Samples collected at the specified times and fixed for fluorescence microscopy. B) BY4741 cells carrying the pMsn2-GFP construct were grown to exponential phase (A_{660} 0.8) on YPD or YP containing ethanol (2%) as carbon source, shifted to pH 8.0. The subcellular distribution of the Msn2-GFP fusion was monitored for at least 200 cells per time point. Open bars denotes fully cytoplasmic, closed bars, nuclear/cytoplasmic, and crossed bars, fully nuclear localization. Data shown correspond to a representative experiment. Three independent experiments were performed with similar results.

Figure 4. Effect of lack of Msn2 and Msn4 transcription factors on high pH tolerance. A) Upper panel. Wild type W303-1A alone (WT) was plated along with strain MCY5278, a W303-1A derived strain lacking both *MSN2* and *MSN4* genes. Lower panel, strain MCY5278 was transformed with centromeric plasmid YCp22 (which carries a *TRP1* gene marker, see main text for explanation) and plated with the equivalent strain but lacking all three *TPK* genes [14]. Dilutions of the cultures were grown for 2 days at the indicated pH. B) Strain W303-1A (WT) and the *msn2 msn4* derivative transformed with the empty plasmid YCp50 or the same plasmid carrying a hyperactive *RAS2*^{Ala18Val19} allele (*RAS2**). Dilutions of the cultures were spotted on YPD plates at the indicated pHs and growth monitored after 2 days. C) Strain W303-1A (open bars) and its *msn2 msn4* derivative (closed bars) carrying a wild type allele of *SNF1* (+) or a *snf1* deletion (-) were grown at the indicated pHs. Growth is denoted as the percentage compared to the same strains cultured at pH 5.5. Data are mean ± SEM from two independent experiments performed by triplicate.

Figure 5. Alkaline pH stress induces STRE-mediated transcriptional activation. A) The wild type strain DBY746 was transformed with episomal plasmids pPHO84-LacZ (pPHO84) and pAMS366 (pCDRE). These strains, together with strain AGS66 (carrying a STRE(7x)-LacZ reporter system integrated at the *URA3* locus) were grown and subjected to high pH stress (pH 8.0). β-galactosidase activity was measured as described and it is represented as percentage over the maximum value for each strain. B) Wild type strain W303-1A (continuous lines) and its *msn2 msn4* derivative (discontinuous lines) were transformed with the *HXK1*-LacZ reporter. Cultures grown on YP (plus 4% glucose) were subjected to high pH (8.0, triangles) or high temperature (37 ºC, squares) and samples taken at different times for β-galactosidase activity measurements. Non-induced cultures are denoted by circles. In all cases, data correspond to means ± SEM from 6 independent experiments.

Figure 6. Msn2/Msn4 dependence of the transcriptional response to alkaline stress. Upper panel, number of genes induced in the wild type and the *msn2 msn4* strain after 10 or 30 min of alkaline stress, deduced from the DNA microarray analysis. Lower panel, evaluation of the Msn2/Msn4 level of dependence of the transcriptional response to high pH after 10 min (open bars) and 30 min (closed bars). Ind, Independent; WD, weakly dependent; SD, strongly dependent; TD, totally dependent. See main text for definitions.

Figure 7. The dependence of *ENA1* expression on the presence of Msn2/Msn4. A) Fold-change of *ENA1* expression deduced from microarray data for W303-1A wild type cells (WT, empty bars) and

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MCY5278 (*msn2 msn4*, filled bars) exposed to pH 8.0 for the indicated times. Data are mean ± SEM from 4 microarray experiments. B) Strains with the indicated genotype (+, wild type allele; -, deletion mutant) were transformed with plasmid pKC201, which bears a *LacZ* fusion of the *ENA1* promoter. Exponential cultures were resuspended in medium buffered at pH 5.5 (empty bars) or pH 8.0 (filled bars) for one hour and β-galactosidase activity measured. Data are mean ± SEM from 6 to 9 independent experiments.

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TABLE 1. Yeast strains used in this work. Except otherwise indicated, single BY4741-derived *kanMX* disruptans not listed here correspond to the systematic gene disruption project [25].

Name	**Relevant genotype**	**Source / reference**
BY4741	*MATa his3Δ1 leu2Δ met15Δ ura3Δ*	[25]
MAR231	BY4741 *bcy1::kanMX4*	This work
W303-1A	*MATa ade2-1, can1-100, his3-112, leu2-3, trp1-1, ura3-1*	[52]
SC7	W303-1A *ira1::LEU2*	[42]
SC8	W303-1A *ira2::URA3*	[42]
PM903	W303-1A *ira1::LEU2 ira2::URA3*	[42]
WΔN1	W303-1A *cdc25::CDC25 aa907–1589/URA3*	[53]
WΔN2	W303-1A *cdc25::CDC25 aa1147–1589/URA3*	[53]
CCV35	W303-1A *pde1::kanMX4*	This work
PM942	W303-1A *pde2::URA3*	[27]
CCV36	W303-1A *pde1::kanMX4 pde2::URA3*	This work
MCY5278	W303-1A *msn2::kanMX4 msn4::hphMX4*	Gift from M. Carlson
CCV174	W303-1A *nrg1::nat1*	This work
CCV175	W303-1A *msn2::kanMX4 msn4::hphMX4 nrg1::nat1*	This work
tpk123 msn2 msn4	W303-1A *tpk1::URA3 tpk2::HIS3 tpk3::TRP1 msn2::HIS3 msn4::TRP1*	[14]
CCV37	W303-1A *snf1::LEU2*	This work
CCV38	W303-1A *msn2::kanMX4 msn4::hphMX4 snf1::LEU2*	This work
BY4742	*MATα his3Δ1 leu2Δ met15Δ ura3Δ*	[25]
DC90	BY4742 *tpk1::kanMX::HIS3 tpk2^{w3} tpk3::LEU2*	J.M. Thevelein
DBY746	MAT*a ura3-52 leu2-3112 his3-1 trp1-239*	D. Botstein
AGS66	DBY746 *URA3*-STRE(7x)-*lacZ*	[29]

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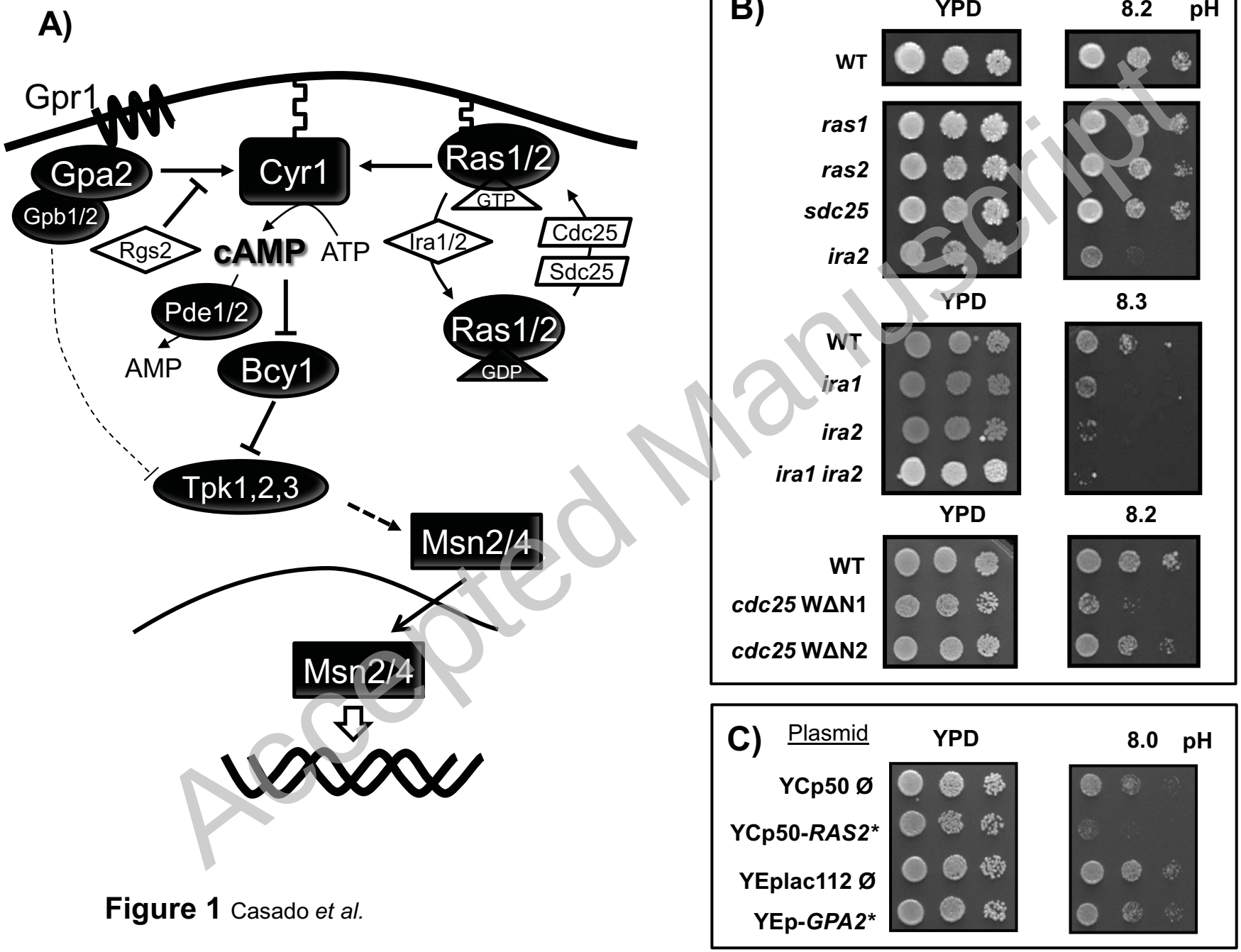

Figure 1 Casado *et al.*

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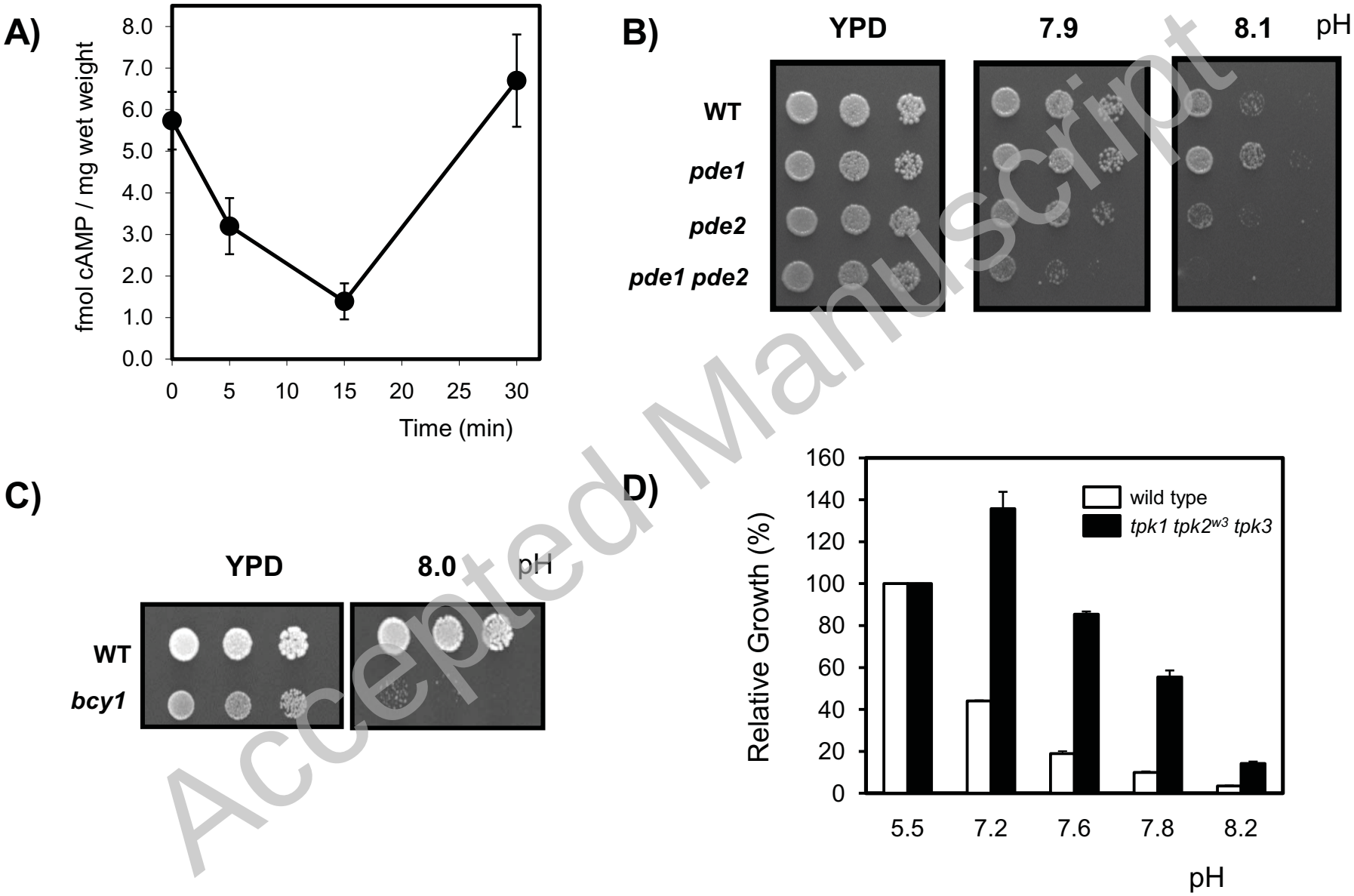

Figure 2 Casado *et al.*

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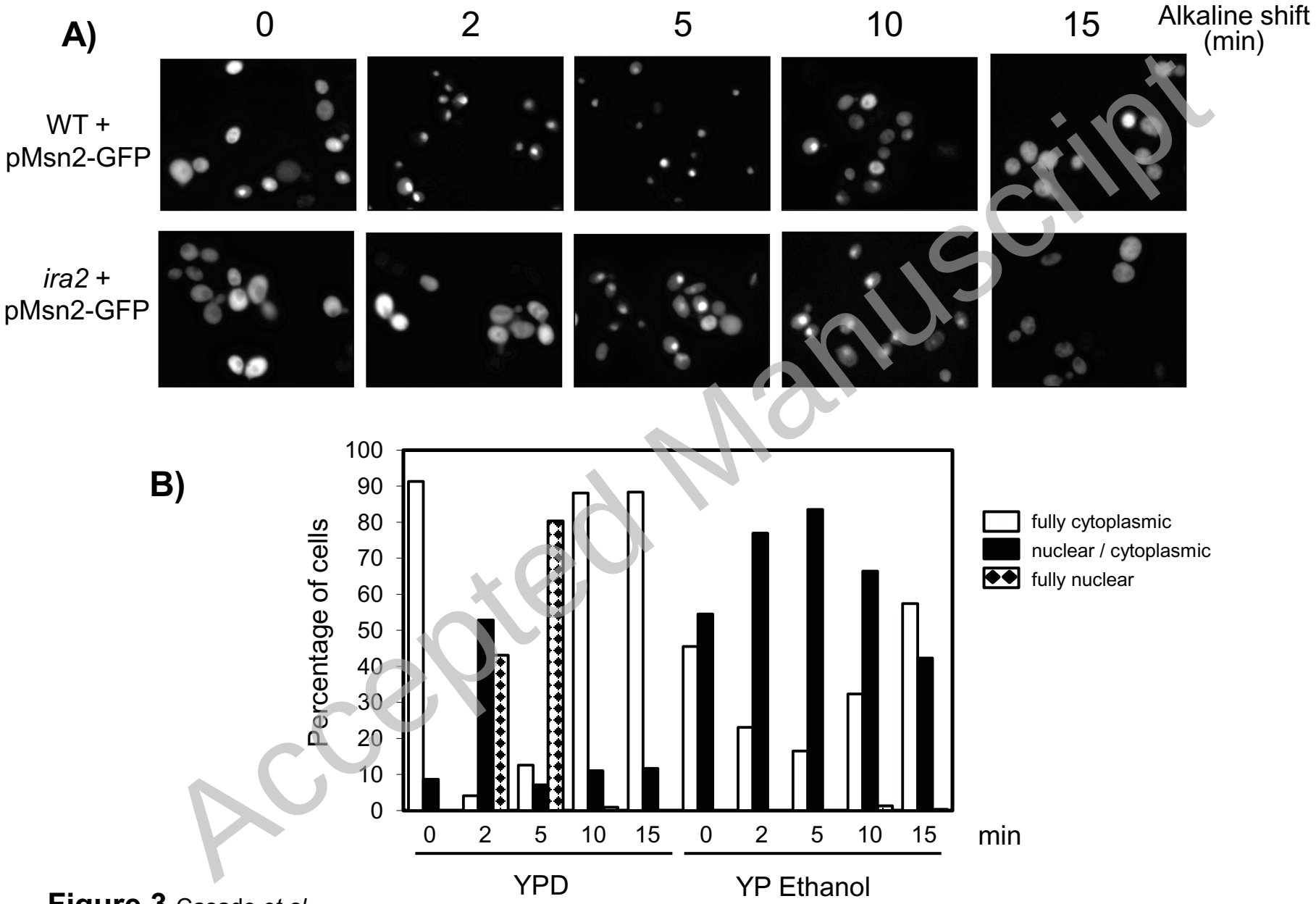

Figure 3 Casado *et al.*

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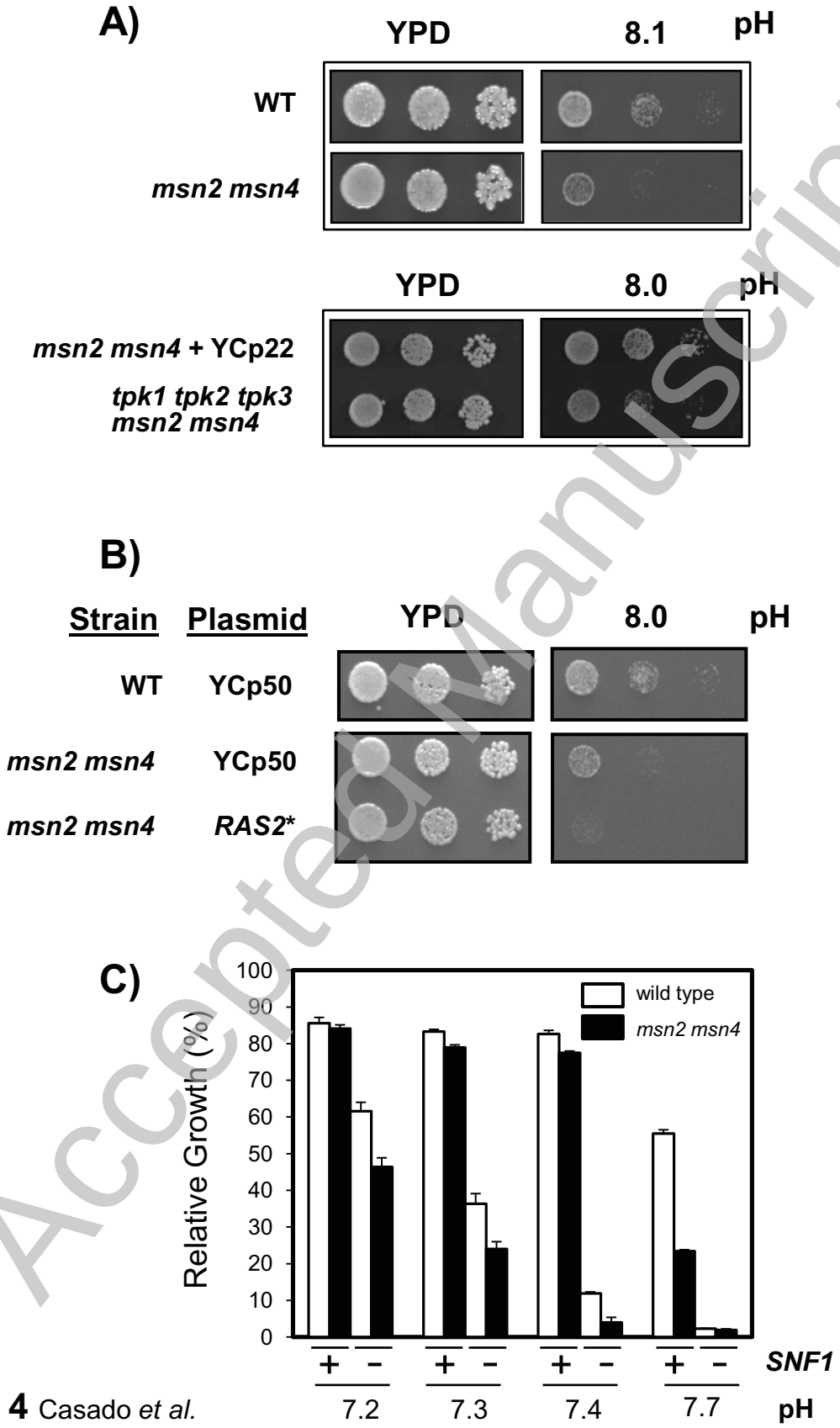

Figure 4 Casado *et al.*

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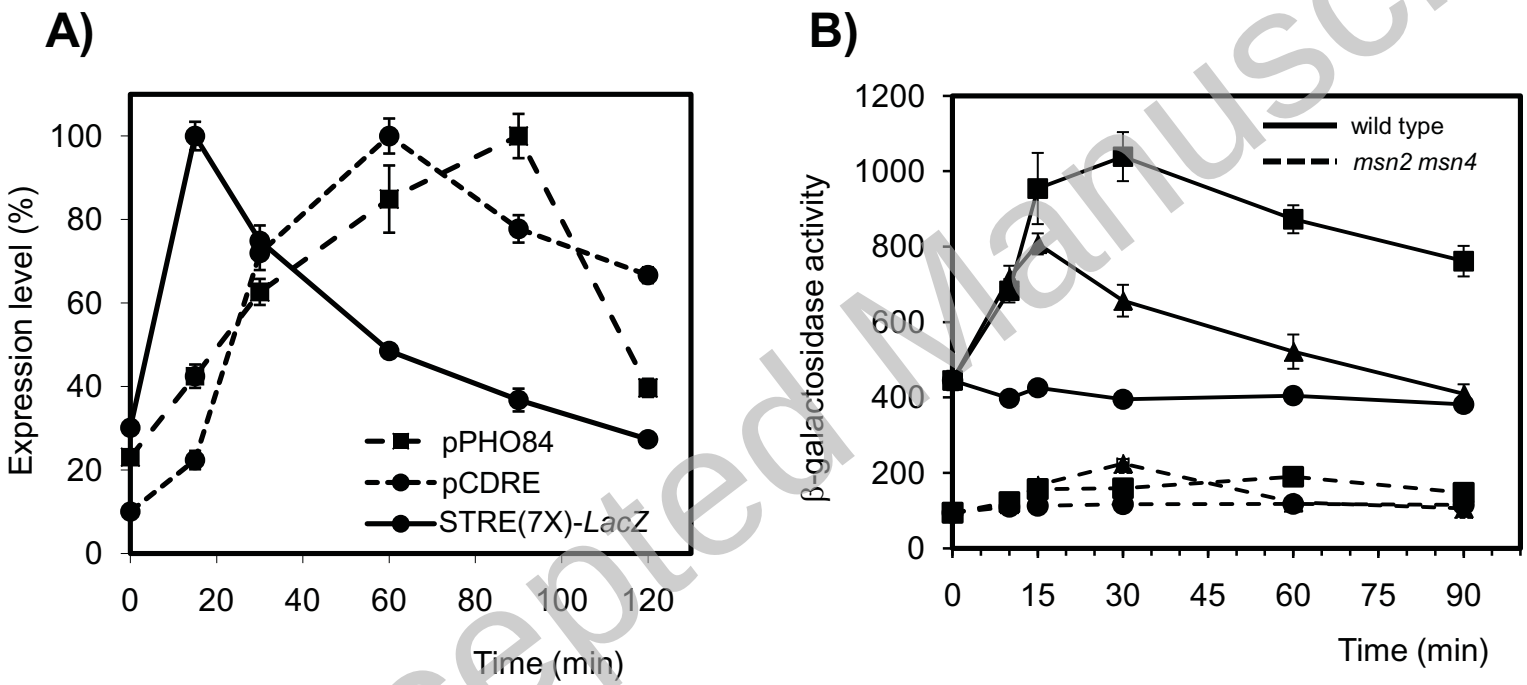

Figure 5 Casado *et al.*

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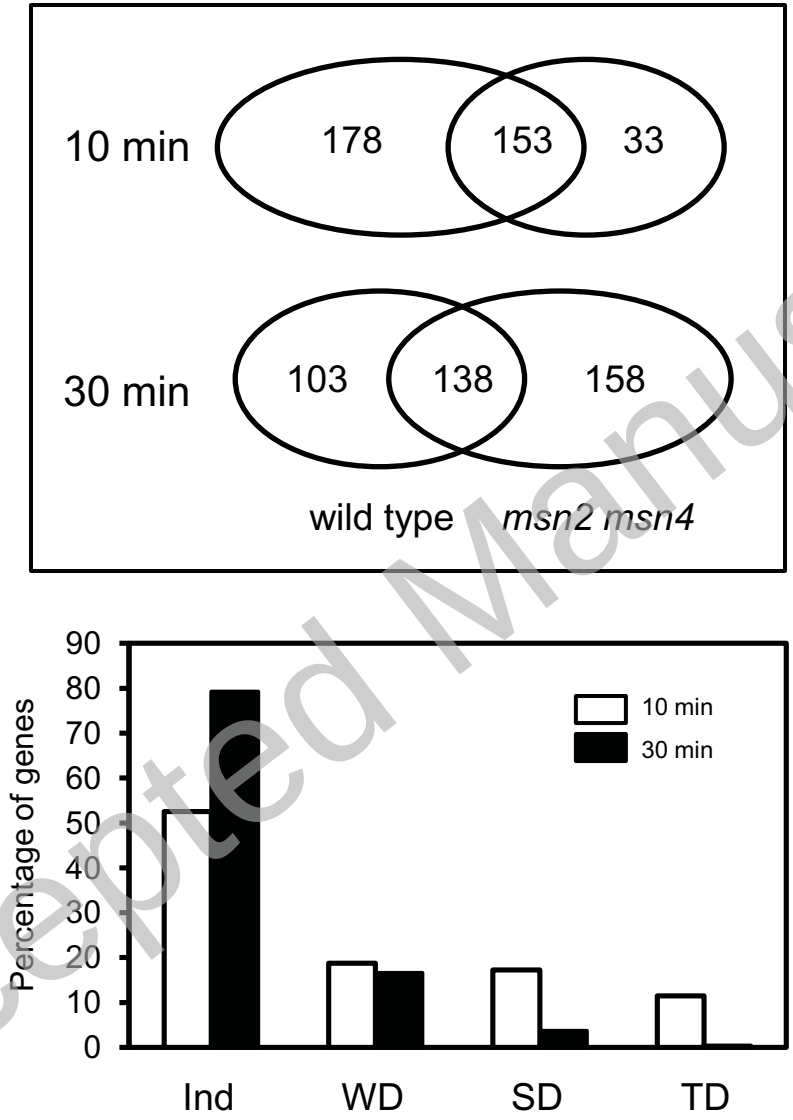

Figure 6 Casado *et al.*

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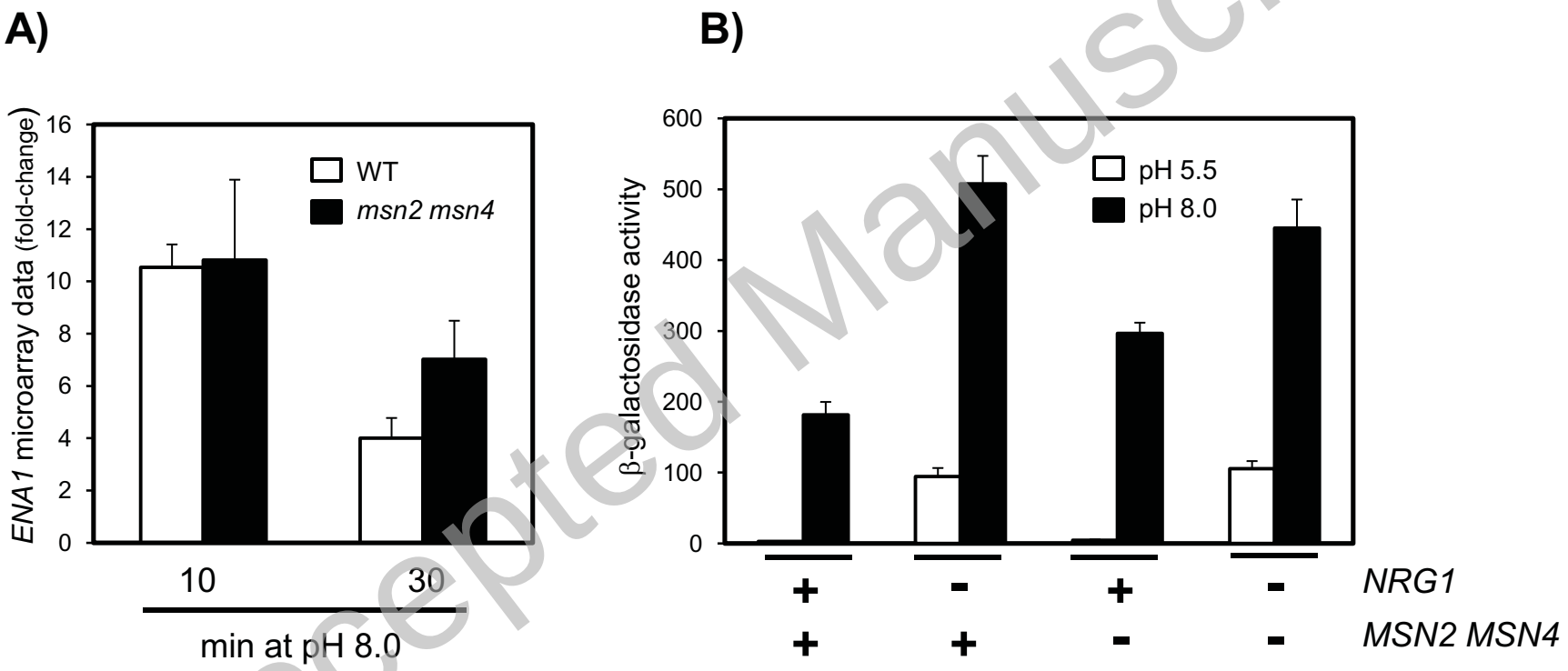

Figure 7 Casado *et al.*

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