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Ref2, a regulatory subunit of the yeast protein phosphatase 1, is a novel component of cation homeostasis

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Short title: Ref2 influences cation homeostasis

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Abbreviations footnote: *CDRE*, Calcineurin Dependent Response Element, RLU, Relative Luminiscence Units

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SYNOPSIS

Maintenance of cation homeostasis is a key process for any living organism. Specific mutations in Glc7, the essential catalytic subunit of yeast protein phosphatase 1, result in salt and alkaline pH sensitivity, suggesting a role for this protein in cation homeostasis. We screened a collection of Glc7 regulatory subunit mutants for altered tolerance to diverse cations (Na^+ , Li^+ , Ca^{+2}) and alkaline pH. Among eighteen candidates, only deletion of *REF2* yielded increased sensitivity to these conditions, as well as to diverse organic toxic cations. A Phe-374 to Ala mutated version of Ref2, which cannot bind Glc7, is unable to rescue the salt-related phenotypes of the *ref2* strain, suggesting that Ref2 function in cation homeostasis is mediated by Glc7. The *ref2* deletion mutant displays a marked decrease in lithium efflux, which can be explained by the inability of these cells to fully induce the Na^+ -ATPase *ENA1* gene. The effect of lack of Ref2 is additive to that of blockage of the calcineurin pathway and might disrupt multiple mechanisms controlling *ENA1* expression. *ref2* cells display a striking defect in vacuolar morphogenesis, which probably accounts for the increased calcium levels observed under standard growth conditions and the strong calcium sensitivity of this mutant. Remarkably, the evidence collected indicates that the role of Ref2 in cation homeostasis may be unrelated to its previously identified function as a component of the APT complex.

Introduction

An elevated intracellular concentration of sodium ions is harmful for most eukaryotic cells, probably because it interferes with the correct functioning of certain cellular targets [1]. Yeasts, as well as other single-cell eukaryotic organisms, have developed mechanisms to maintain a safe cytosolic concentration of this cation even when the external concentration of sodium ions in their natural environments is high. To this end, these organisms employ three distinct strategies: a) discrimination among different alkali metal cations at the level of uptake (favouring transport of potassium vs sodium), b) triggering the efficient efflux of toxic cations, and c) sequestering the excess of cations in organelles, such as the vacuole.

The budding yeast *Saccharomyces cerevisiae* is a model organism used very often for cation tolerance studies. In this organism, the capacity to extrude sodium (or other toxic cations, such as lithium) represents a major mechanism to maintain low intracellular levels of these toxic cations. This is achieved by two different and complementary ways. First, the function of an H^+/Na^+ antiporter encoded by the *NHA1* gene, which is able to extrude Na^+ , Li^+ , and even K^+ cations by exchange with protons and, therefore, acquires biological relevance at rather acidic external pHs [2;3]. The second mechanism is based on a P-type ATPase pump encoded by the *ENA* system (see [4] for a recent review). In *S. cerevisiae* the *ENA* genes are disposed in tandem repeats with a variable number of copies (*ENA1-ENA5*) encoding identical or very similar proteins [5-7]. Deletion of the entire *ENA* cluster in *S. cerevisiae* results in a dramatic phenotype of sensitivity to sodium and lithium cations or alkaline pHs [5;6;8;9]. It is widely accepted that *ENA1* is the functionally relevant component of the cluster on the basis that 1) cation sensitivity is largely restored by expression of *ENA1* [6;10] and, 2) whereas under standard growth conditions expression from the *ENA* genes is almost undetectable, *ENA1* expression dramatically increases in response to osmotic, saline or alkaline pH stress [6;8;9;11].

Regulation of *ENA1* expression in response to saline or alkaline stress is under the control of different pathways [12]. These include the HOG osmoresponsive MAP kinase Hog1, the calcium-activated protein phosphatase calcineurin, the Rim101/Nrg1, the Snf1 and the Hal3/Ppz1 pathways, among others (see [4] and references therein). Activation of calcineurin upon saline or alkaline pH stress results in dephosphorylation and nuclear entry of the Crz1 transcription factor [13-15], which binds to specific sequences, known as CDRE, in calcineurin-responsive promoters. Two CDREs have been defined in the *ENA1* promoter (-821/-813 and -727/-719) the downstream element being the most relevant for induction of the gene in cells exposed to saline or high pH stress [16-19].

Protein phosphatase 1 is a conserved Ser/Thr protein phosphatase whose catalytic subunit is encoded in *S. cerevisiae* by a single, essential gene, named *GLC7* [20;21]. The Glc7 protein is required for a myriad of cellular functions and specific regulation of these functions is achieved by interaction of the catalytic subunit with a variety of regulatory proteins, which influence Glc7 intracellular localization and/or substrate recognition. Many of these regulatory subunits interact with Glc7 through a conserved binding site, which usually appears as a (R/K)(V/I)X(F/W) consensus sequence [22;23]. A search of currently available *S. cerevisiae* databases (i.e. *Saccharomyces* Genome Database, <http://www.yeastgenome.org/>) reveals at least 119 proteins that are annotated as Glc7 interacting proteins. However, most of these relationships are based on recent high-

throughput analysis, whereas compelling regulatory evidence has been only collected for a limited subset of these proteins.

Among the cellular functions attributed to the Glc7 phosphatase, a role in ion homeostasis was postulated some years ago as a result of the characterization of the *glc7-109* allele [24], which carried a mutation at Arg-260 found to be responsible for the mutant phenotypes. *glc7-109* mutants displayed, among other phenotypes, a marked sensitivity to Na^+ cations and alkaline pH, which was remedied by inclusion of potassium ions in the medium. Interestingly, Arg-260 lies in the vicinity of Phe-256, a residue in the Glc7 hydrophobic channel that makes contact with the aromatic residue of the (R/K)(V/I)X(F/W) motif present in many regulatory subunits. Therefore, we hypothesized that the defect in ion homeostasis in the *glc7-109* mutant could be due to a deficient interaction of the mutated protein with a regulatory subunit relevant for ion tolerance. To this end, we have analyzed the tolerance to diverse cations of eighteen known or putative Glc7 regulatory subunits and found that deletion of *REF2* yields a rather severe and unique phenotype of sensitivity to Na^+ , Li^+ and alkaline pH. *REF2* was initially described as a gene encoding a protein required for mRNA 3'-end maturation prior to the final polyadenylation step [25]. Evidence for interaction between Ref2 and Glc7 was obtained by both two-hybrid and affinity purification techniques [26-28]. Subsequently, it has been found that Ref2 is a component of the APT (for Associated with P_TA1) subcomplex of the large cleavage and polyadenylation factor (CPF)-containing complex (holo-CPF), which is required for formation of mRNA and snoRNA 3'-ends and also contains Glc7 [29]. It has been shown that Ref2 is required for the permanence of Glc7 within the complex [30]. The Ref2 protein contains a ³⁶⁸RISSIKFLD³⁷⁶ sequence that resembles the Glc7 binding motif and recent evidence indicates that mutation of Ref2 Phe-374 to Ala disrupts the interaction between this protein and Glc7, thus preventing incorporation of Glc7 to the APT subcomplex [30]

In this work, we highlight the phenotype of cation sensitivity of the *ref2* mutant and investigate the molecular basis of this defect. Our results indicate that mutation of *REF2* results in a deficient efflux of toxic cations that can be, at least in part, attributed to impaired expression of the *ENA1* ATPase gene. Remarkably, this role on salt tolerance seems unrelated to the function of Ref2 in the APT complex.

Experimental

Growth of *Escherichia coli* and yeast strains

Escherichia coli DH5 α cells were used as plasmid DNA host and were grown at 37 °C in LB supplemented with 50 μ g/ml ampicillin when plasmid selection was required. Yeast cells were cultivated at 28 °C in YPD medium (10 g/l yeast extract, 20 g/l peptone, and 20 g/l dextrose) or in synthetic complete drop-out medium when carrying plasmids. Restriction reactions, DNA ligations and other standard recombinant DNA techniques were performed as described elsewhere [31]. Yeast cells were transformed using the lithium acetate method [32]. Sensitivity of yeast cells to lithium, sodium or calcium chloride and alkaline pH was evaluated by growth on plates (drop tests) or in liquid cultures as previously described [8;33]. Growth under limiting potassium concentration was carried out using an YNB-based medium (Translucent K-free medium, prepared by Formedium, UK; manuscript in preparation) formulated so that potassium content in the final medium is negligible (~15 μ M).

Gene disruption and plasmid construction

Single *KanMX* deletion mutants in the BY4741 background (*MATa his3 Δ 1 leu2 Δ met15 Δ ura3 Δ*) were generated in the context of the *Saccharomyces* Genome Deletion Project [34]. Replacement of the *REF2* coding region by the *nat1* marker from *Streptomyces noursei* was accomplished as follows. The 1.40 kbp DNA fragment containing the *nat1* gene flanked by genomic sequences corresponding to -40 to -1 and +1603 to +1643 from the *REF2* ATG codon was amplified from the plasmid pAG25 [35] with the oligonucleotides OJFD021 and OJFD022 (Supplemental Table 1) and transformed in the wild type strains BY4741 and its *cnb1::KanMX* derivative to yield strains YJFD1 and YJFD7, respectively. The *ref2::nat1* mutation was also introduced in the wild type DBY746 background (*MATa, ura3-52 leu2-3,112 his3 Δ 1 trp1 Δ 239*) to yield strain YJFD5, which was used for intracellular calcium levels measurements. Positive clones were selected in the presence of 100 μ g/ml nourseothricin (Werner BioAgents, Germany) and the presence of the deletions was verified by PCR. Strains YJFD17 and YJFD18 were made by co-transformation of wild type BY4741 and its *ref2::KanMX* derivative, respectively, with plasmid pJFD10 (containing the *glc7-109* allele, see below) and a disruption cassette containing the *nat1* gene flanked by genomic sequences corresponding to -41 to -1 and +1465 to +1509 from the *GLC7* ATG codon (amplified from plasmid pAG25 with the oligonucleotides OJFD81 and OJFD82). Diverse tests were carried out to ensure that the only Glc7-encoding gene copy corresponded to the plasmid-borne *glc7-109* allele. Strains KT1112 (*GLC7*), KT1935 (*glc7-109*), and KT2210 (*glc7-F256A*), a generous gift from K. Tatchell, were previously reported [24]. Strains SB6 (*YTH1*) and SB7 (*yth1-1*) [36]; YWK168 (*SWD2*) and YWK172 (*swd2-3*) [37] as well as W303 (*SSU72*) and YWK186 (*ssu72-2*) [38] were a generous gift from B. Ditchl. Strains PTA1 (*PTA1*) and *pta1* (*pta1 Δ 1-75*) [39] were kindly provided by C. Moore.

To clone the *glc7-109* allele in the YCplac33 plasmid (centromeric, *URA3* marker) the Glc7-encoding ORF flanked by 2068 bp upstream and 148 bp downstream was amplified by PCR using genomic DNA from strain KT1935 and oligonucleotides Glc7-BamHI.Up and Glc7-PstI.Do, which contain artificial BamHI and PstI restriction sites,

respectively (Supplemental Table 1). The amplified DNA fragment was digested by BamHI and PstI and cloned in the same restriction sites of YCplac33 to yield plasmid pJFD10 (YCplac33-*gic7*-109). The plasmid was isolated and sequenced.

For low-copy, centromeric expression of *REF2*, a 2.28 kbp fragment containing the *REF2* ORF flanked by 500 bp and 200 bp from its promoter and terminator regions, respectively, was amplified using a specific pair of oligonucleotides containing artificially added BamHI (OJFD027) and SacI (OJFD026) restriction sites (Supplemental Table 1). The resulting DNA fragment was digested with BamHI and SacI and cloned in the same restriction sites of the pRS415 plasmid (centromeric, *LEU2* marker) to yield pRS415-*REF2* (pJFD1).

To generate the F374A allele of *REF2* (pJFD2), the pRS415-*REF2* construct was used as a PCR template to change the TTT codon, encoding a phenylalanine residue at position 374, to a GCT codon, encoding an alanine residue. Two overlapping DNA fragments (a and b) were generated. The 1.75 bp fragment **a**, spanning from nt -501 to +1140 (relative to the initiating Met) was obtained using oligonucleotides OJFD029 and M13-20 reverse. The 0.7 bp length fragment **b** comprises from nt +1101 to +1785 and was amplified with oligonucleotides OJFD063 and M13-20 (Supplemental Table 1). To allow *in vivo* DNA-recombination, these two fragments were co-transformed in yeast cells together with the linearized pRS415 plasmid digested with BamHI and SacI. Cells carrying the pRS415^[F374A] construct were selected in a medium lacking leucine and the plasmid was recovered and sequenced to ensure the absence of unexpected mutations.

LacZ reporter plasmids used in this work have been reported previously. *ENA1* reporters were pKC201, containing the entire *ENA1* promoter [40] and pMRK212 and pMRK213, containing the ARR1 and ARR2 regions of this same promoter [17]. pMRK-based constructs derive from plasmid pSLFA-178K [41], which contains a *CYC1* minimal promoter and displays virtually no reporter activity in the absence of a transcriptionally active DNA insert. Plasmid pAMS366 [14] contains four copies of a CDRE from the *FKS2* promoter.

Lithium content and lithium efflux measurements.

Lithium content and efflux were measured as previously reported with minor modifications [42]. For determination of the intracellular Li⁺, cells were grown overnight in liquid YPD medium. When cultures reached an optical density of 0.5-0.6, cells were collected and diluted (A_{600} of 0.2-0.3), in fresh YPD medium containing the appropriate concentration of LiCl. Cells were loaded with lithium during 8 h and then samples were filtered, washed with 20 mM MgCl₂, and treated with acid. Lithium was analyzed by atomic absorption spectrophotometry as previously described [43]. Li⁺ efflux was studied in cells grown overnight as described above. Cells were suspended in fresh medium and loaded with 75 mM LiCl for two hours. Yeast cells were separated from the culture medium by centrifugation, washed, and suspended in fresh lithium-free medium. At time intervals, samples were taken and treated as above for lithium determination.

β-Galactosidase Assays

The different strains were transformed with the reporter plasmids pKC201, pMRK212, pMRK213 or pAMS366. Cultures were grown up to saturation in the appropriate dropout medium, then inoculated on YPD (pH 5.5) until A_{660} 0.6-0.7 was reached and centrifuged (5 min at 750 x g). Then cells were resuspended in fresh YPD adjusted to pH 5.5 containing the indicated concentrations of LiCl, NaCl or CaCl_2 , or adjusted to pH 8.2 in the presence of 50 mM TAPS buffer, and further incubated for 60 min, unless otherwise stated, at the specified temperatures. β -galactosidase activity was measured as described previously [17].

Intracellular calcium monitoring

The determination of intracellular calcium was based in the use of pEV11/AEQ [44] which carries the *APOAEQUORIN* gene under the control of the *ADH1* promoter. Evaluation of cytoplasmic calcium was carried out essentially as described previously [18;44]. Briefly, DBY746 wild type and *ref2* mutant cells (YJFD5) were transformed with plasmid pEVP11/AEQ and grown to an A_{660} of 1.0. Aliquots of 30 μl of culture were incubated in luminometer tubes for 20 min at room temperature. One-tenth volume of 592 μM coelenterazine (Sigma) in methanol was added to each sample and further incubated at room temperature for 20 min. Basal luminescence was recorded every 0.1 s for 5 s. using a Berthold LB9507 luminometer. The relative luminescence units (RLU/s) were normalized by the number of cells in each sample.

Vacuolar staining and visualization

Vacuole morphology was assessed basically as described previously [45]. Five ml of yeast cultures at A_{660} of 0.8-1.0 were harvested, washed with YPD, and resuspended in 0.1 ml of fresh YPD. The lipophilic fluorescent dye FM4-64 (Invitrogen) was added at a final concentration of 20 μM and cells were incubated for 15 min at 30 °C. The cells were then washed, resuspended in 3 ml of YPD, and incubated for 15-45 min to allow the internalization by endocytosis and accumulation of the dye within the vacuole. Cells were visualized with a FITC filter using a Nikon Eclipse E800 fluorescence microscope (400 \times). Digital images were captured with an ORCA-ER 4742-80 camera (Hamamatsu) using the Wasabi software.

Results

Lack of Ref2 causes defects in cation tolerance

The observation that Glc7 point mutations lying in the vicinity of residues relevant for binding diverse regulatory subunits may cause alterations in ion homeostasis prompted us to examine tolerance to NaCl, LiCl, alkaline pH and calcium in eighteen strains carrying a deletion of functionally well-documented regulatory subunits of Glc7. Figure 1 shows a selected panel of conditions. As it can be observed, mutation of most regulatory subunits does not result in significantly altered tolerance to the indicated treatments, with the exception of the *ref2* and *shp1* mutants. However, deletion of *shp1* caused a rather severe growth defect even in the absence of stress, as previously described [46], making difficult to assess the actual effect of the mutation under the conditions tested. In contrast, lack of Ref2, which only caused a slight growth defect under standard culture conditions, prevented growth in the presence of lithium, sodium and calcium cations, as well as under alkaline conditions. *ref2* cells were also markedly sensitive to caesium ions (not shown). The intensity of the phenotype for Na⁺ and Li⁺ stress was comparable to that of a *trk1 trk2* mutant (not shown) and it was not caused by the inability to adapt to hyperosmotic stress, since 1) growth was not affected by inclusion of 1 M sorbitol or KCl in the medium and 2) an obvious growth defect was already observed at a lithium concentration as low as 30 mM, which does not represent an osmotic stress condition (data not shown). Increased sensitivity to Na⁺ and Li⁺ cations is often observed in strains with defective potassium uptake (i.e. *trk1* mutants). This prompted us to test the requirements for potassium in the *ref2* mutant, by using a recently developed YNB-based K-free medium. As observed in Figure 2, *ref2* cells display a slow-growth phenotype at limiting potassium concentrations (0.5-1.5 mM) that was alleviated by increasing the amount of potassium in the medium. This suggests that *ref2* cells might have a decreased high-affinity potassium uptake, although direct measurement of rubidium influx (an analogue commonly used to assess for potassium uptake capacity) failed to reveal a significant decrease in Rb⁺ uptake (data not shown). In any case, the *ref2* strain showed increased sensitivity to several organic toxic cations, such as hygromycin B, spermine and tetramethylammonium (TMA), a phenotype which is commonly observed in strains with altered cation homeostasis (Figure 3A).

Ref2 contains a RISSIKFLD sequence (residues 368-376) that closely matches the (R/K)(V/I)X(F/W) Glc7-binding consensus sequence. We considered it necessary to test whether or not the salt-related phenotypes derived from the absence of Ref2 could be explained by its role as putative Glc7-regulatory subunit. To this end, Phe-374 was changed to Ala and the mutated version of the gene (including its own promoter) was cloned in a centromeric plasmid. As shown in Figure 3A, introduction of the wild type version of *REF2* (pJFD1) in a *ref2* mutant was able to increase tolerance to sodium, lithium and alkaline pH, as well as to diverse toxic organic cations. In contrast, mutation of Phe-374 (pJFD2) resulted in complete inability to do so. It must be noted that this mutation does not affect Ref2 cellular levels but it is known to disrupt the interaction with Glc7 [30]. Expression of the mutated form of Ref2 from a high-copy number plasmid, which should lead to overexpression of the protein, was also unable to rescue the *ref2* defects (not shown). These evidences suggest that blocking interaction of Ref2 with Glc7 results in loss of Ref2 function in salt tolerance. A similar effect was observed when the growth deficiency at limiting external potassium

concentrations of the *ref2* mutant was tested (Figure 3B). In this case, expression of the Phe-374 mutated version of *REF2* (pJFD2) even aggravated the growth defect at low potassium of the strain lacking the chromosomal copy of the gene. We then considered the possibility that the cation-related defects of the *ref2* strain could be attenuated or even suppressed by a surplus of Glc7. However, expression of Glc7 on a multicopy plasmid did not increase at all tolerance to LiCl or NaCl of a *ref2* strain (Supplementary Figure 1). In contrast, the same plasmid was able to abolish the salt sensitive phenotype of specific Glc7 mutants (Supplementary Figure 1), indicating proper expression of Glc7.

Increased sensitivity to Na⁺ and Li⁺ can be the result of increased uptake, decreased efflux, or inability to sequester these toxic cations into intracellular compartments, such as the vacuole. To get insight into a possible cause for sensitivity in the *ref2* mutant, the intracellular concentration of lithium in cells grown in the presence of 25 or 50 mM LiCl was tested. As observed in Figure 4A, *ref2* mutants accumulate 60 to 70% more lithium than the wild type strain. This suggested that lack of Ref2 might favour lithium entry or make detoxification of the cation more difficult by interfering with the efflux systems. To this end, we tested the ability of *ref2* cells to extrude lithium. As shown in Figure 4B, lithium efflux was markedly impaired in cells lacking Ref2, suggesting that the absence of this Glc7 regulatory subunit interferes with the normal efflux mechanisms.

Mutation of Ref2 affects expression of the *ENA1* ATPase gene under saline and alkaline pH stresses.

The Na⁺-ATPase *Ena1* is a major determinant of sodium and lithium tolerance. The expression of the gene is dramatically increased by exposure to stressing concentrations of Li⁺ or Na⁺ or alkalinization of the medium, and many mutations impairing *ENA1* induction upon stress have been shown to lead to hypersensitivity to these cations. Therefore, we considered it necessary to monitor the expression of *ENA1* in *ref2* cells under cation stress conditions. To this end, cells were transformed with plasmid pKC201, which carries the entire *ENA1* promoter fused to the *lacZ* reporter gene. As shown in Figure 5A, expression driven from the *ENA1* promoter in cells exposed to Na⁺ or Li⁺ cations, as well as to alkaline pH, is drastically reduced in *ref2* cells. Therefore, the increased sensitivity of the *ref2* mutant to these stresses could be attributable, at least in part, to a defect in *ENA1* induction.

The expression of *ENA1* under saline or alkaline stress is controlled by a variety of signalling pathways, including activation of calcineurin, which is triggered by a burst of cytosolic calcium. As observed in Figure 5A, the effect of the *ref2* mutation on *ENA1* expression is even more dramatic than that of mutation of *CNB1*, encoding the regulatory subunit of calcineurin. Furthermore, the *cnb1* mutation seems additive to *ref2* both with respect to *ENA1* expression (Figure 5A) or Li⁺, Na⁺ and alkaline pH tolerance (Figure 5B). Therefore, *ref2* phenotypes cannot be exclusively explained by a hypothetical impairment of calcineurin signalling under stress. Most regulatory inputs acting on the *ENA1* promoter target two relatively small regions named ARR1 and ARR2 (see [4] for a recent review). To gain insight into the effect of the *ref2* mutation, cells were transformed with plasmids pMRK212 and pMRK213. The former contains a region which mostly integrates calcium/calcineurin inputs, whereas the latter is regulated by calcineurin-independent stimuli. As shown in Figure 5A, deletion of *REF2* decreases expression driven from both

promoter regions, suggesting a complex and diverse effect of the mutation. Analysis of the expression from these constructs further supports an additive effect of the *cnb1* and *ref2* mutations on cation homeostasis.

***ref2* cells display a hyperactivated calcium/calcineurin pathway in the absence of stress and show altered vacuole morphology**

A striking phenotype that can be observed in Figure 1 is that lack of Ref2 results in a substantial sensitivity to calcium cations. It has been reported that hyperactivation of calcineurin results in a calcium-sensitive phenotype, whereas deletion of calcineurin-encoding genes yields calcium hypertolerance. We then hypothesized that the calcium-sensitive phenotype of *ref2* mutants could be attributed to a higher-than-normal basal calcineurin activity (that is, in the absence of saline or alkaline pH stress). To test this possibility, we evaluated calcium tolerance in cells lacking both Ref2 and Cnb1, in comparison with the single *ref2* and *cnb1* mutants. As shown in Figure 6A, the calcium sensitivity conferred by the *ref2* mutation is largely abolished in the absence of Cnb1. This suggests that the *ref2* growth defect in the presence of calcium is caused by hyperactivation of calcineurin and leads to the possibility that calcium levels might be higher-than-normal in *ref2* cells. This was directly tested by introducing the calcium-reporter plasmid pEV11/AEQ in wild type and *ref2* mutant cells. Our measurements indicated that the luminescence observed in *ref2* cells was 141.9 ± 3.7 RLU/ 10^6 cells, whereas this parameter was 64.8 ± 3.5 in the wild type strain, thus confirming the existence of higher free cytosolic calcium levels in the mutant. On the basis of this result, one would expect that expression from a specific calcium/calcineurin-sensitive promoter would be increased in *ref2* cells grown under basal (non-stressing) conditions. To test this conjecture, wild type and *ref2* strains (as well as their *cnb1* derivatives) were transformed with plasmid pAMS366, which carries a tandem of four copies of the CDRE from the promoter of the *FKS2* gene. As shown in Figure 6B, under basal growth conditions, expression from this synthetic promoter is 4 to 5-fold higher in *ref2* cells than in the wild type strain. As expected, this effect is abolished in the absence of calcineurin activity (*cnb1 ref2* strain). Remarkably, while in the wild type strain addition of calcium ions to the medium triggers a dramatic, calcineurin-mediated increase in expression, this effect is much less prominent in cells lacking Ref2. This could be explained if the *ref2* mutation somehow interferes with stress-triggered effects mediated by activation of calcineurin. Increased sensitivity to alkaline pH and calcium is a characteristic phenotype of yeast strains with deficient vacuolar function. Therefore, the possibility that *ref2* mutants may display some alteration in this subcellular compartment was considered. As shown in Figure 6C, incubation of wild type and *ref2* cells with the lyophilic fluorescent dye FM4-64, which stains the vacuole membrane, reveals that vacuolar structure is dramatically altered in the *ref2* mutant, with a punctuated fluorescent pattern and lack of discernible vacuolar structure. Because of the similarity between the saline phenotypes of the *ref2* deletion and the *glc7-109* allele, we considered it interesting to evaluate vacuolar morphology in the latter. As observed in Figure 6C, FM4-64 staining of strain YJFD17, which carries a centromeric plasmid-borne *glc7-109* allele as only source of *GLC7* function reveals that this strain displays vacuoles with essentially wild-type morphology (similarly to the original cation-sensitive strain KT1935, not shown). Interestingly, combination of the *ref2* deletion and the *glc7-109* mutation results in cells with *ref2* phenotype, that is, altered vacuoles. The disruption of *REF2* in the KT1935 background was repeatedly attempted without success.

This is likely due to the close genetic relationship of this strain with the W303 genetic background (K. Tatchell, personal communication), in which the *REF2* deletion was reported to be lethal [47].

Mutants in components of the APT complex do not share the cation-related phenotypes of the *ref2* strain.

Given the previously described function of Ref2 as a component of the APT complex, it was reasonable to consider that the striking cation-related phenotypes of the *ref2* strain could be attributed to the role of the protein in this complex. We reasoned that, if so, mutations in components of the complex should yield phenotypes reminiscent of those of the *ref2* strain. Since many members of the APT complex are essential (including Pta1), we had to resort in most cases to temperature sensitive (*ts*) mutants cultured at sub-lethal temperatures. Figure 7A shows that deletion of *SYC1*, encoding a non-essential member of the complex, does not alter cell growth under conditions that clearly affect proliferation of *ref2* mutants. The *yth1-1* mutant displayed a slight sensitivity to LiCl, but was indistinguishable from the wild type in its sensitivity to NaCl and alkaline pH. The *swd2-3* and *ssu72-2* strains did not show any sensitivity to LiCl or NaCl and only a marginal sensitivity to high pH. None of these strains displayed sensitivity to high calcium levels in the medium. Similarly, a *ts* strain lacking the N-terminal region of Pta1 (strain *pta1* Δ 1-75) exhibited a near wild type tolerance to cations or alkaline pH even at 37 °C. We confirm here the phenotype of sensitivity to formamide attributed to the *yth1-1* strain when grown at 30 °C [36]. Interestingly, we observe a similar behaviour for the *swd2-3* mutant at 30 °C or the *ref2* and *pta1* Δ 1-75 strains at 37 °C, clearly showing that under these conditions the function of the APT complex is compromised. The almost complete lack of overlap between specific *ref2* phenotypes and those of other mutants composing the APT complex is exemplified in the phenotypic array shown in Figure 7B.

We also examined the basal expression level of *ENA1* in these strains and the ability of the *ENA1* promoter to be activated by salt stress. As shown in Figure 8, changes in expression levels induced by exposure to LiCl or NaCl were smaller in the *swd2-3* strain. However, the expression observed in *syc1*, *ssu72-2* or *pta1* Δ 1-75 derivatives was virtually identical to that of their respective wild type strains. Taken together, these results demonstrate that the mutation of diverse components of the APT complex does not mimic the cation-related phenotypes of the *ref2* strain.

Discussion

In this work we present a screen for defects in cation tolerance of eighteen strains lacking specific regulatory subunits of the yeast type 1 protein phosphatase Glc7. Among them, only the *ref2* mutant presented phenotypes undoubtedly associated to altered cation homeostasis, such as decreased tolerance to lithium and sodium, sensitivity to alkaline pH and decreased growth at limiting external potassium. We also observe that *ref2* cells are sensitive to diverse toxic organic cations which interfere with very different cellular functions. It is generally accepted that these compounds enter the cell driven by the electrochemical gradient generated by the Pma1 plasma membrane proton ATPase and it has been observed that many mutations that affect Na⁺ and/or K⁺ homeostasis result in altered electrochemical gradient (i.e. *trk1*, *ppz1*, *hal4,5*, ...). Therefore, the observation that *ref2* cells are sensitive to hygromycin B, spermine and TMA is consistent with the notion that Ref2 is a relevant component of cation homeostasis. Our data suggest that the decreased tolerance to alkaline cations of the *ref2* mutant could be due, at least in part, to the inability to fully induce expression of the *ENA1* Na⁺-ATPase gene, which is a major determinant in saline tolerance. Since lack of Ref2 blocks the response of *ENA1* to diverse stresses that are mediated through a variety of signalling pathways [12;48], the effect of Ref2 on *ENA1* expression is possibly pleiotropic and may involve multiple targets.

Ref2 and Glc7 are components of the APT complex and they are required for efficient 3' processing of mRNAs and transcription termination of certain snoRNA genes ([25;29;30;47]. Therefore, it would be reasonable to speculate that the cation-related phenotypes of the *ref2* mutant could be caused by malfunction of this complex, which is itself a component of the holo-CPF. However, our results show that deletion or *ts* mutations of diverse components of the APT complex, tested under conditions that impair their function in the complex [37-39], do not mimic consistently the growth defects or impaired *ENA1* expression of the *ref2* strain (Figure 7B and 8). The same situation is observed for the conditional mutation of *YTH1*, whose product participates in polyadenylation factor I, a different subcomplex of the CPF [36]. Furthermore, we also observe that while the *ref2* strain fails to grow on non-fermentable carbon sources and grows poorly on low glucose, none of the APT-related mutations tested display such phenotypes (Figure 7B and Supplementary Figure 2), thus providing an additional example of disparate phenotypes. On the other hand, Ref2 has been related to the COMPASS/Set1 complex through the Swd2 protein [29]. The function of Swd2 in this complex has been shown to be independent from its role in APT complex [37]. The COMPASS/Set1 complex is required for Histone H3 methylation at K4, which is a fingerprint of actively transcribed genes [49]. Therefore, defective COMPASS function could be invoked to explain the effect of the *ref2* mutation on *ENA1* expression. However, this seems to be unlikely since: 1) a survey of the literature (and our own results, not shown) indicates that mutants in the non-essential components of the complex do not display salt-related phenotypes nor are they sensitive to organic toxic cations; 2) no evidence for a role of Glc7 on COMPASS function has been reported so far, and 3) deletion of *REF2* does not perturb Histone H3 methylation at K4 [50].

Our observation that *ref2* cells are calcium-sensitive is consistent with the identification of *REF2* in a screen for mutations sharing multiple *pmr1* phenotypes [51]. *PMR1* encodes an ATPase that sequesters Ca⁺² and Mn⁺² into the Golgi/secretory pathway

that is pivotal to maintain proper cytosolic concentrations of these ions. In addition, similarly to *vma* and *pmr1* mutants [51], the *ref2* mutant displays strong sensitivity to the cell-wall perturbing agent calcofluor white (data not shown). Remarkably, we observe in *ref2* cells grown in the absence of any source of stress increased cytosolic calcium levels and higher-than-normal expression from a synthetic calcium/calcineurin-regulated promoter (Figure 6B). This suggests that basal calcineurin activity is abnormally high in *ref2* mutants. Further activation of calcineurin caused by exposure to high concentrations of extracellular calcium would explain the hypersensitive phenotype of *ref2* cells to this cation, since it is known that excessive calcineurin activity is detrimental for the cell [52;53]. The observation that deletion of *CNB1*, which blocks calcineurin signalling, relieves calcium toxicity provides further support to this hypothesis. We show here that *ref2* cells display altered vacuolar morphology, which is reminiscent of the class B/C *vps* mutants [45;54;55]. Since the vacuole is the most important calcium store in yeast [56], it is reasonable to assume that the calcium-related phenotypes displayed by *ref2* cells could be derived from their incapacity for normal vacuole assembly. It must be noted, however, that whereas lack of Ref2 increases basal calcineurin activity, this mutation also interferes with stress signals that transiently activate the phosphatase, as deduced from the lower-than-normal short-term response of the calcineurin-regulatable promoter (Figure 6B).

We show here that a version of Ref2 that cannot interact with Glc7 [30] is unable to rescue the saline phenotypes of the *ref2* strain. Therefore, it is reasonable to consider that the perturbed cation homeostasis observed in a *ref2* strain is due to a deregulation of Glc7 activity. Altered cation homeostasis has been described in the past for specific mutations of Glc7, such as the *glc7-109* allele [24], which carries an Arg-260 to Ala mutation near the hydrophobic channel that is likely to be responsible for interaction with the binding motif present in Ref2. Remarkably, the *glc7-109* mutant displays a subset of phenotypes that resembles very much those reported here for the *ref2* disruption, such as enhanced sensitivity to sodium, lithium, alkaline pH and organic toxic cations [24]. We have also observed that deletion of *REF2* in a strain carrying the *glc7-109* allele (YJFD18) does not result in enhanced sensitivity to NaCl or alkaline pH (not shown). In addition, the saline phenotypes of the *glc7-109* allele are additive to those caused by disruption of calcineurin activity, a circumstance also observed for the *ref2* strain (Figure 5B). All this evidence suggests that the cation-related phenotypes of the *glc7-109* allele could be caused, at least in part, by its incapacity to interact with Ref2. It must be stressed, however, that *ref2* and *glc7-109* phenotypes are not always consistent. For instance, the *glc7-109* strain hyperaccumulates glycogen [24], whereas the *ref2* mutant does not (data not shown). More importantly, the *glc7-109* strain does not display altered calcium levels and increased calcium sensitivity [24], nor altered vacuolar morphology (Figure 6C). Therefore, it could be hypothesized that the R260A mutation in *glc7-109* may affect interaction of the phosphatase catalytic subunit with diverse regulatory subunits, one of these being Ref2. The relevance of the role of Ref2 is highlighted by the observation that the salt-related defects of *ref2* cells cannot be overridden by simply overexpressing Glc7.

In conclusion, our data demonstrates that the role of Ref2 on cation tolerance is not attributable to the function of this protein in regulating Glc7 in the CPF complex but, instead, it suggests that Ref2 must direct Glc7 to alternative target(s). This implies that Ref2 may have multiple cellular functions. While remarkable, this is not fully surprising, since a similar

scenario has been reported for the Gac1 regulatory subunit, which functions as a molecular scaffold to tether Glc7 to Gsy2 (encoding glycogen synthase), thus controlling glycogen accumulation [24], and also binds the transcription factor Hsf1, thus controlling the transcription of certain heat-shock responsive genes, such as *CUP1* [57]. Therefore, Ref2 would represent a novel example of multifunctional Glc7 regulatory subunit.

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LEGENDS

Figure 1. Effect of the mutation of several Glc7-interacting proteins on cation-related phenotypes. Two dilutions ($\sim 3 \times 10^3$ and 3×10^2 cells) from cultures of wild-type strain BY4741 and the indicated isogenic derivatives were grown on YPD plates in the presence of 0.6 M NaCl, 50 mM LiCl, 0.2 M CaCl_2 or alkaline pH for 2 days.

Figure 2. Mutation of *REF2* increases potassium requirements for growth. Cultures of wild type strain BY4741 (open bars) and its *ref2* derivative (closed bars) were inoculated at A_{600} of 0.004 in Translucent K-free medium supplemented with the indicated amounts of KCl and grown for 16 h. Results are represented as percentage of growth compared with cells incubated with 50 mM KCl and are mean \pm SEM from 3 independent cultures.

Figure 3. Mutation of Phe-374 within the Glc7-binding consensus sequence abolishes the function of Ref2 in cation tolerance. **A.** Wild type BY4741 (+) and *ref2* strains (-) were transformed with the empty plasmid pRS415 or the same plasmid expressing the wild type Ref2 protein (pJFD1) or the Ref2^{F374A} mutated version (pJFD2). Transformants were spotted on plates adjusted to pH 8.1 or containing LiCl (150 mM), NaCl (1 M), TMA (0.3 M), Spermine (0.6 mM) or Hygromycin B (20 $\mu\text{g/ml}$) and growth was monitored after 3 days, except for LiCl and NaCl plates (6 days). **B.** Wild type and *ref2* strains were transformed with the indicated plasmids and growth was tested at limiting concentration of external potassium (1 mM). Experimental conditions were as described in the legend of Figure 2.

Figure 4. Effects of the *ref2* mutation on intracellular lithium accumulation and efflux. **A.** Wild-type BY4741 cells (open bars) and the *ref2* derivative (closed bars) were incubated with 25 or 50 mM LiCl as described in Experimental procedures, and the intracellular content of lithium ions measured. Data are mean \pm SD from at least six independent determinations. **B.** Strains BY4741 (—○—) and *ref2* (—■—) were loaded with 75 mM LiCl, samples taken at different times and the efflux of the cation determined as described. Results are expressed as percentage of the intracellular Li^+ content for each strain at time zero and are mean \pm SD from at least six independent assays.

Figure 5. Effects of the *ref2* mutation in wild type and *cnb1* strains on the *ENA1* promoter activity. **A.** The indicated strains were transformed with plasmids pKC201, carrying the entire *ENA1* promoter, or plasmids pMRK212 or pMRK213, which contain specific regions of the ATPase gene promoter. See cartoon for details and relevant regulatory elements (MIG, Mig1/2 binding sequence; CRE, cAMP Regulatory Element). Cells received no treatment (NI), or either were shifted to pH 8.2 (pH) or received 0.4 M NaCl (Na) or 0.2 M LiCl (Li) for 1 h. β -galactosidase activity was measured in permeabilized cells as described in Experimental. Data are mean \pm SEM from 8-9 independent transformants. **B.** Three dilutions of the indicated cultures were spotted on plates at the conditions shown and growth recorded after 72 h (LiCl and NaCl) or 48 h (alkaline pH).

Figure 6. Lack of *REF2* results in altered calcium homeostasis and vacuolar morphology. **A.** The indicated strains were grown in the presence or absence of calcium cations (300 mM CaCl_2) and growth was monitored after 72 h. **B.** Yeast strains were transformed with plasmid pAMS366, which contains a tandem of four CDREs, and incubated for 1 h in the absence (empty bars) or presence (closed bars) of 0.2 M CaCl_2 . β -

galactosidase activity data are mean \pm SEM from 6 independent transformants. **C.** Vacuolar staining of wild type BY4741, *ref2::kanMX*, YJFD17 (*glc7-109*) and YJFD18 (*ref2::kanMX glc7-109*) strains with the fluorescent dye FM4-64 was carried out as described in Experimental for 30 min and monitored by fluorescence microscopy (x400).

Figure 7.- Comparison of *ref2* phenotypes with those of diverse APT-related mutants.

A. Cultures ($\sim 3 \times 10^3$ and 3×10^2 cells) of strains described on the left were spotted on plates and grown at the indicated temperatures (right) for three days. YPDF, YPD plus 3% formamide, LiCl was at 100 mM (30 °C) or 150 mM (37 °C). Conditions for NaCl (800 mM), pH (8.2) or CaCl_2 (200 mM) treatments were identical for both temperatures. **B.** Phenotypic array comparing multiple *ref2* phenotypes with those of other APT-related mutants. Growth of each mutant was compared with that of the corresponding wild type strain grown under the same set of conditions. Filled squares denote strong sensitivity; grey squares indicate a mild phenotype and empty squares correspond to wild type behavior. Conditions tested to construct the array were: LiCl (50-200 mM); NaCl (0.4-1.2 M); High pH (8.0-8.3); Ca^{+2} , (100-300 mM CaCl_2), YPGly, YP plus 2% glycerol; YP-EtOH, YP plus 2% Ethanol; YP-LowGlu, YP plus 0.05% Glucose; Hyg B, Hygromycin B (20-60 $\mu\text{g/ml}$); TMA, Tetramethyl ammonium (0.2-0.6 M).

Figure 8.- Expression of *ENA1* under saline stress in diverse APT-related mutants.

The indicated mutants and their corresponding wild type strains were transformed with plasmid pKC201. Stress conditions were as in Figure 5, except that growth temperature was 30 °C for all strains but *PTA1* and *pta1*($\Delta 1-75$), which were grown at 37 °C. Data are mean \pm SEM from 12 independent transformants. Bars at the bottom connect isogenic strains.

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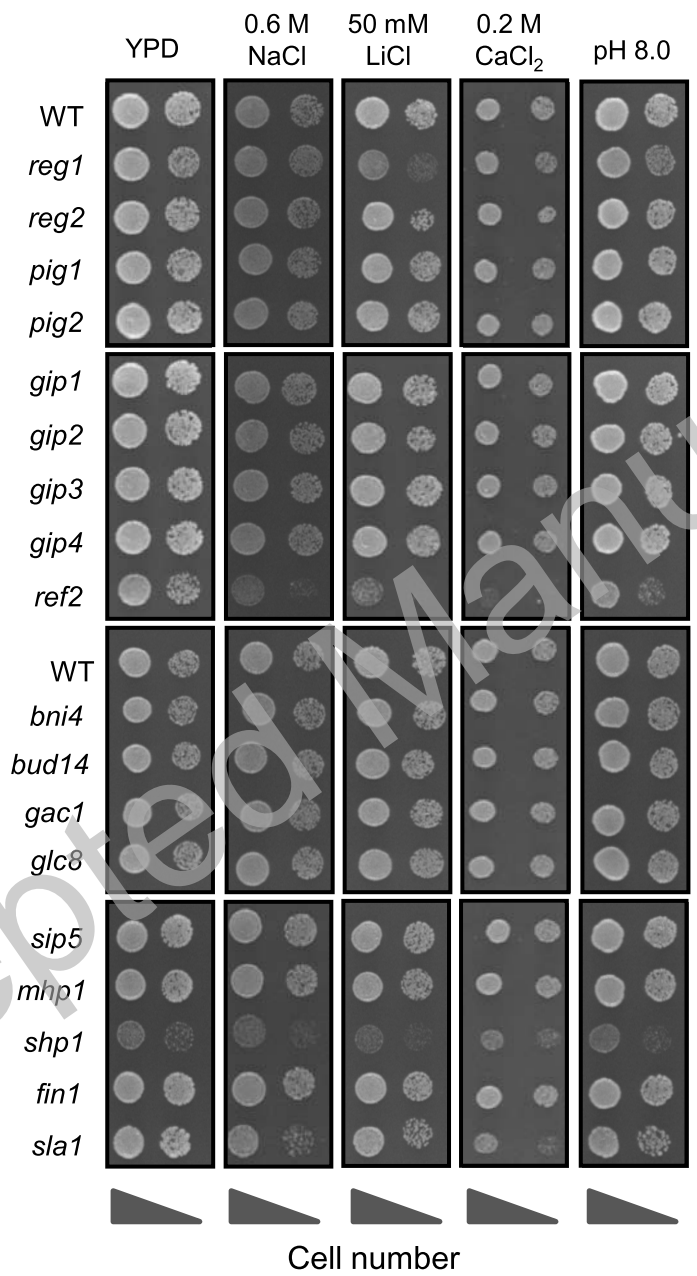


Fig. 1 Ferrer-Dalmau *et al.*

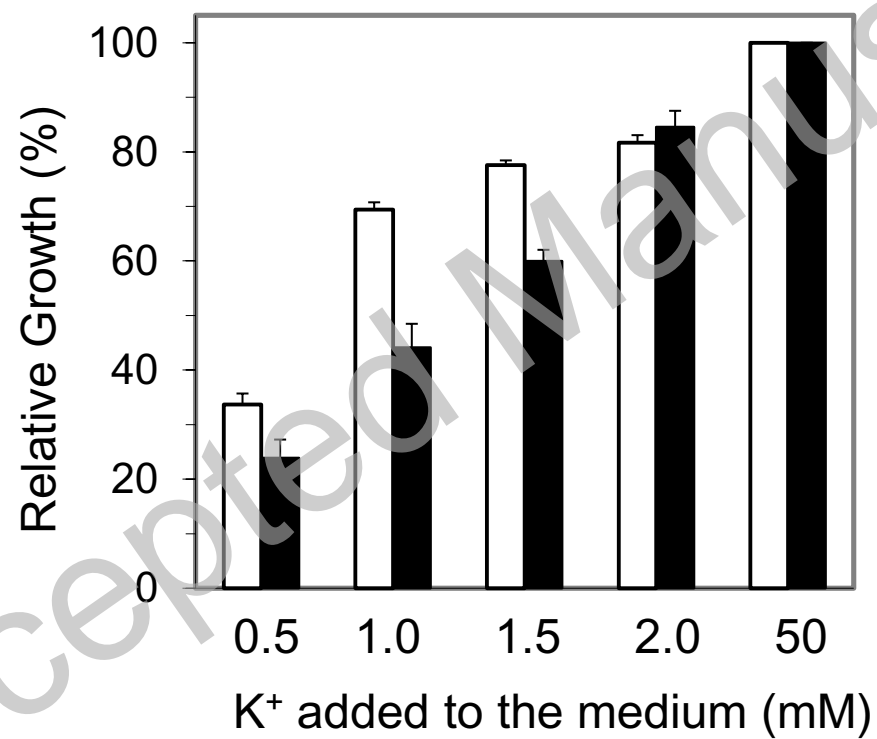


Fig. 2 Ferrer-Dalmau *et al.*

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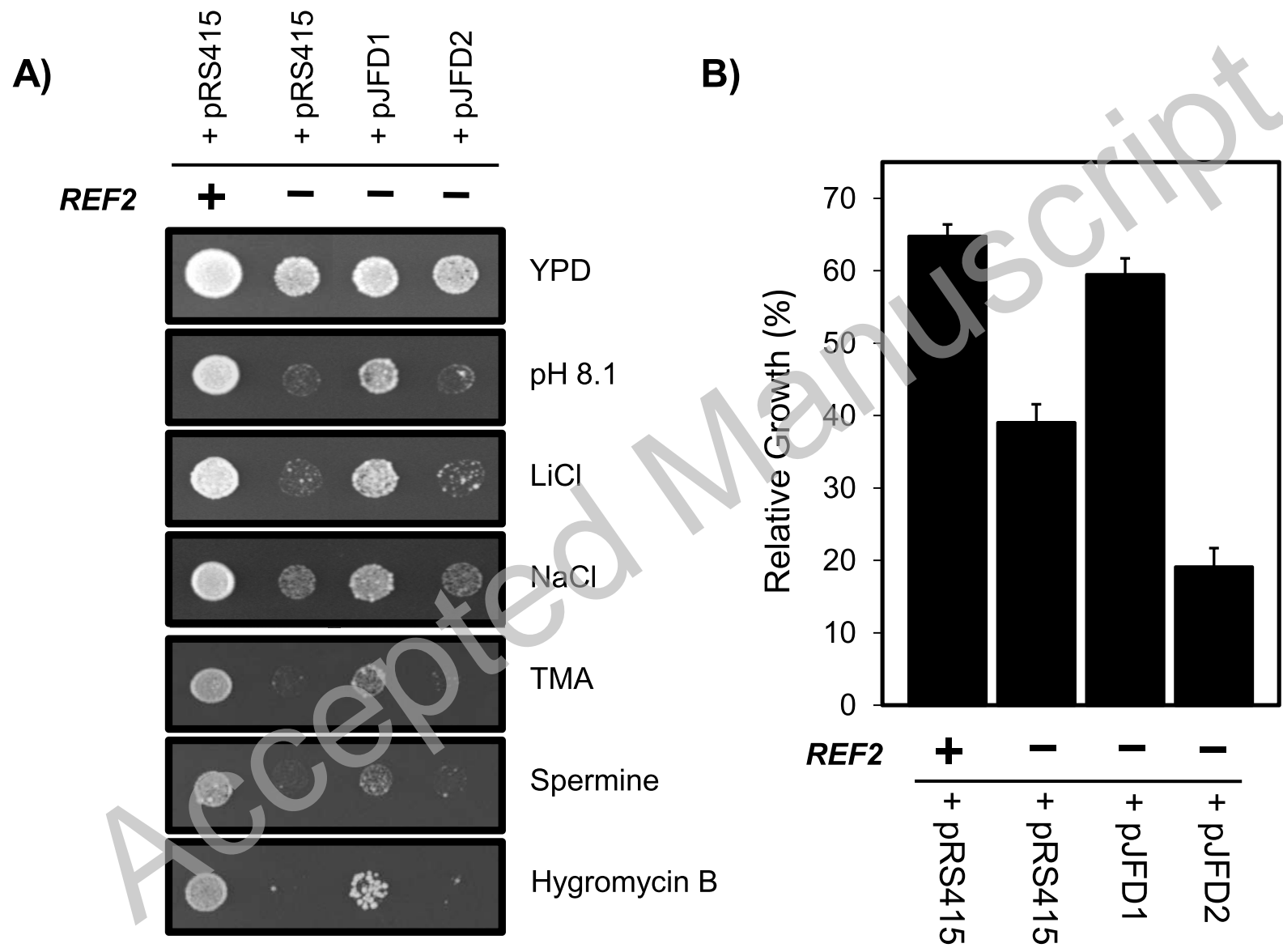


Fig. 3 Ferrer-Dalmau *et al.*

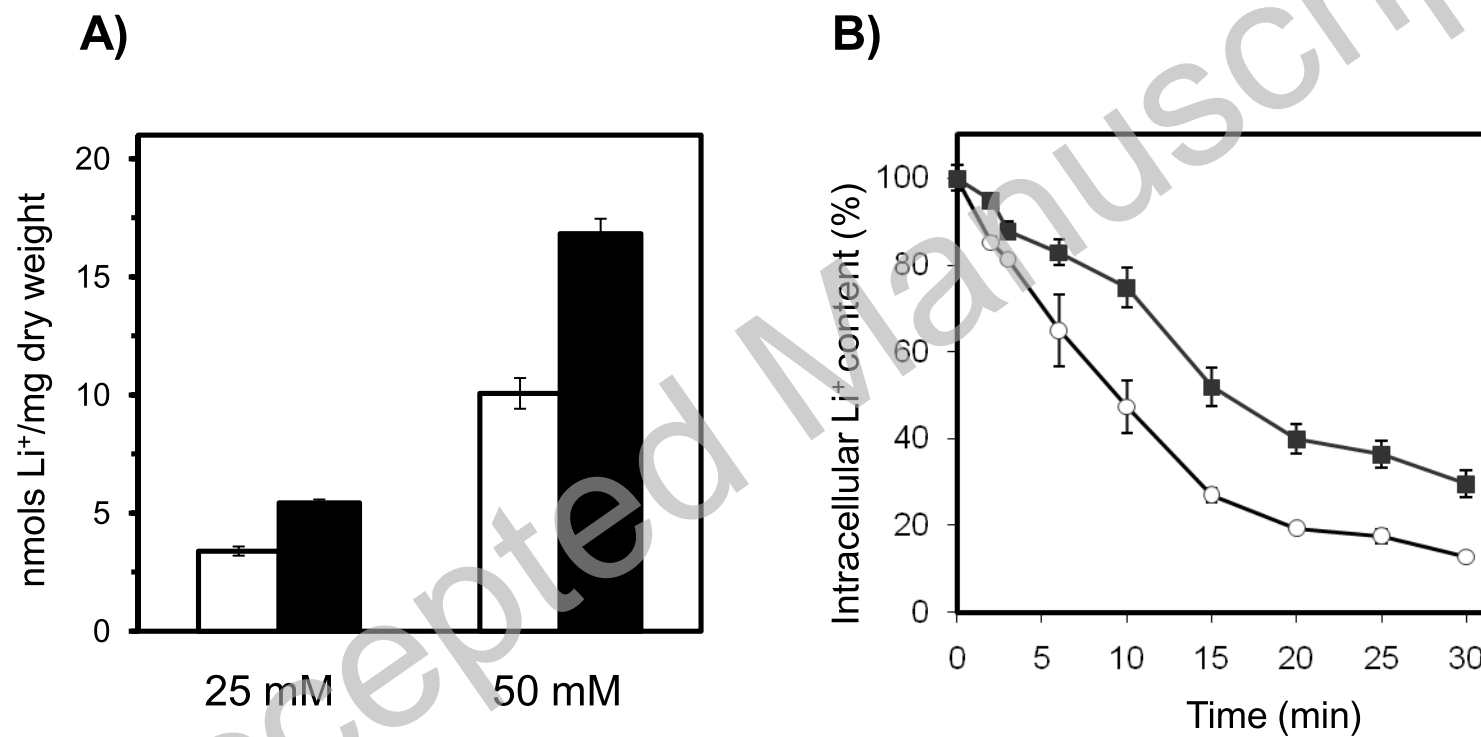


Fig. 4 Ferrer-Dalmau *et al.*

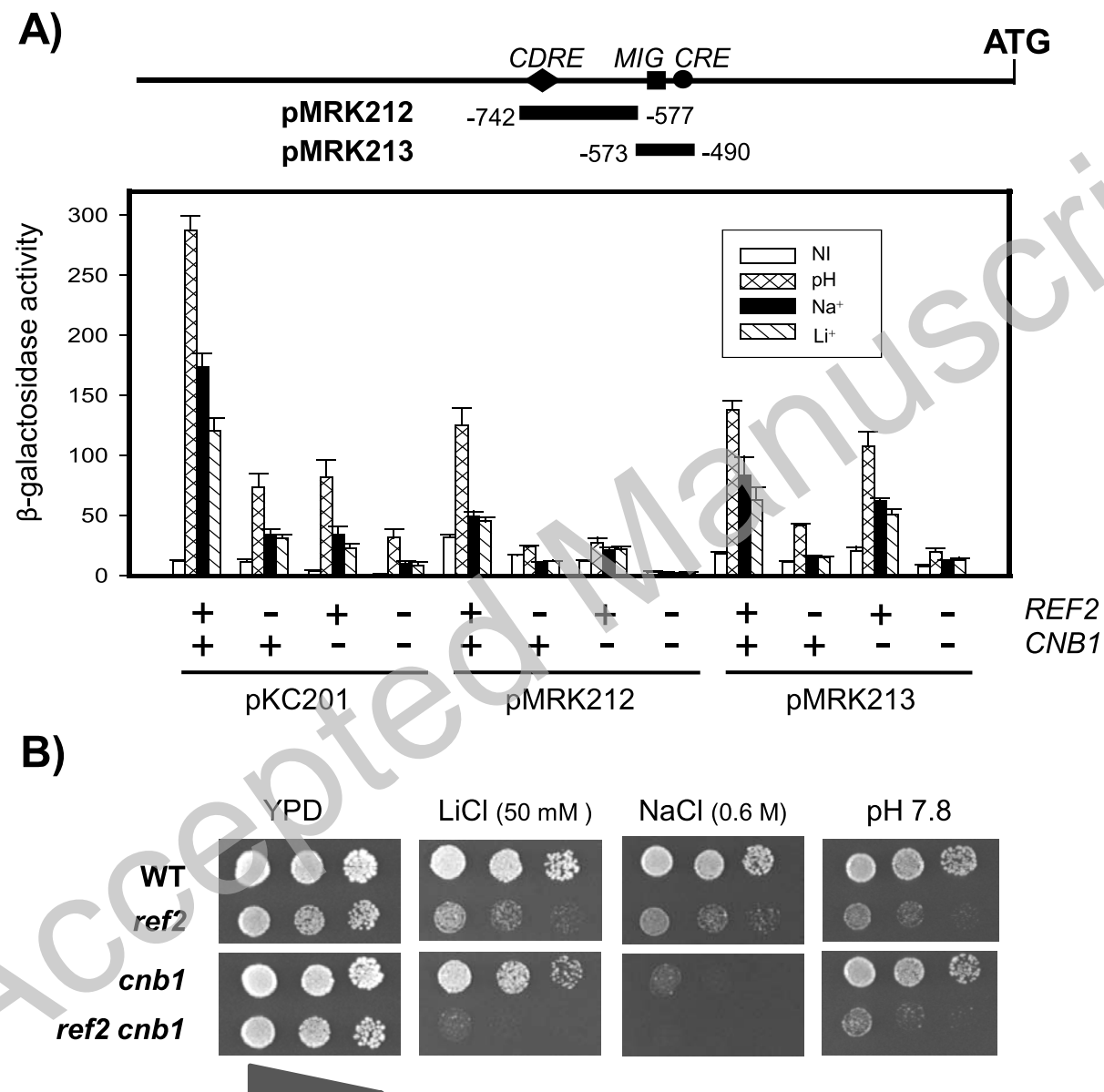


Fig. 5 Ferrer-Dalmau *et al.*

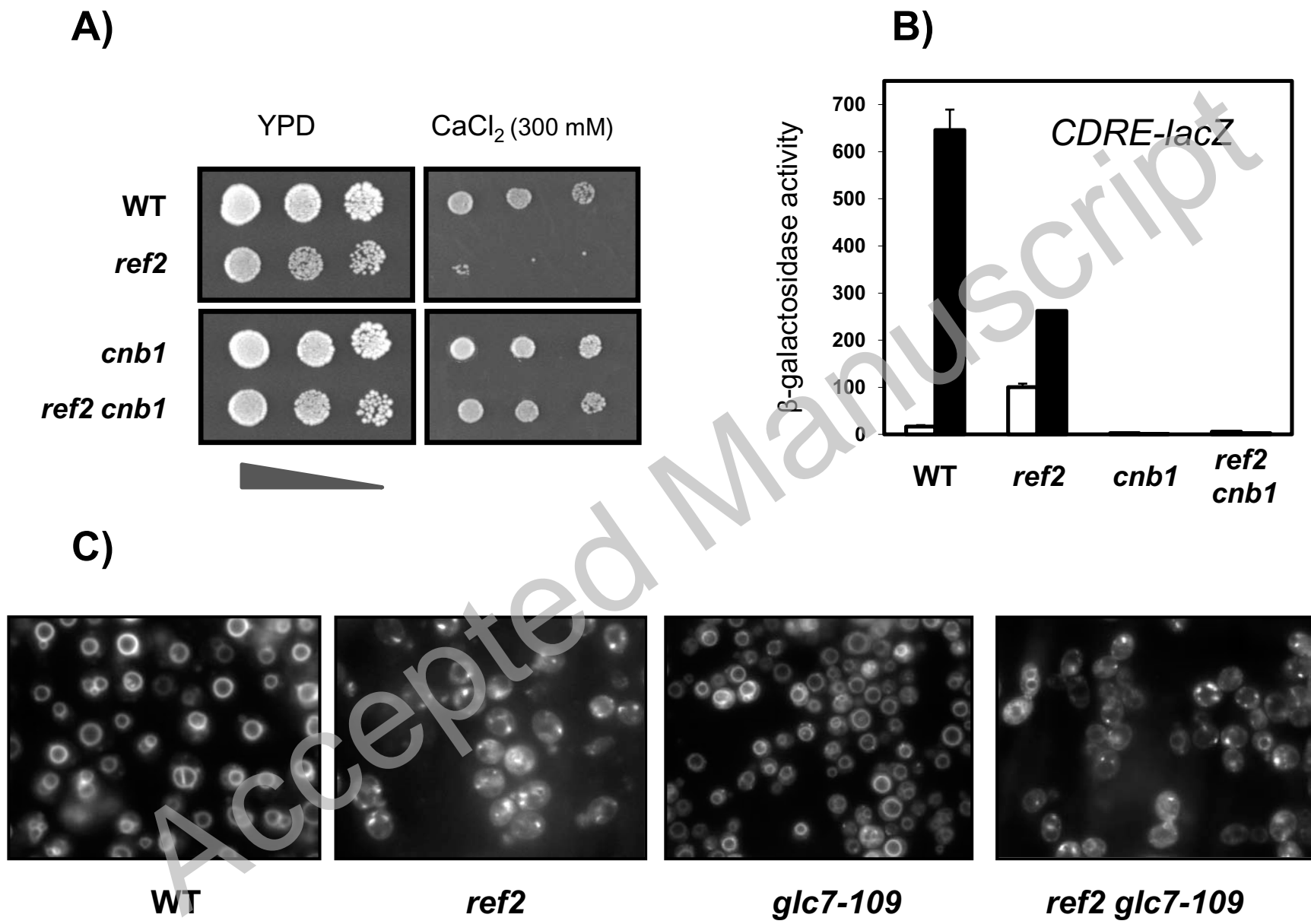


Fig. 6 Ferrer-Dalmau *et al.*

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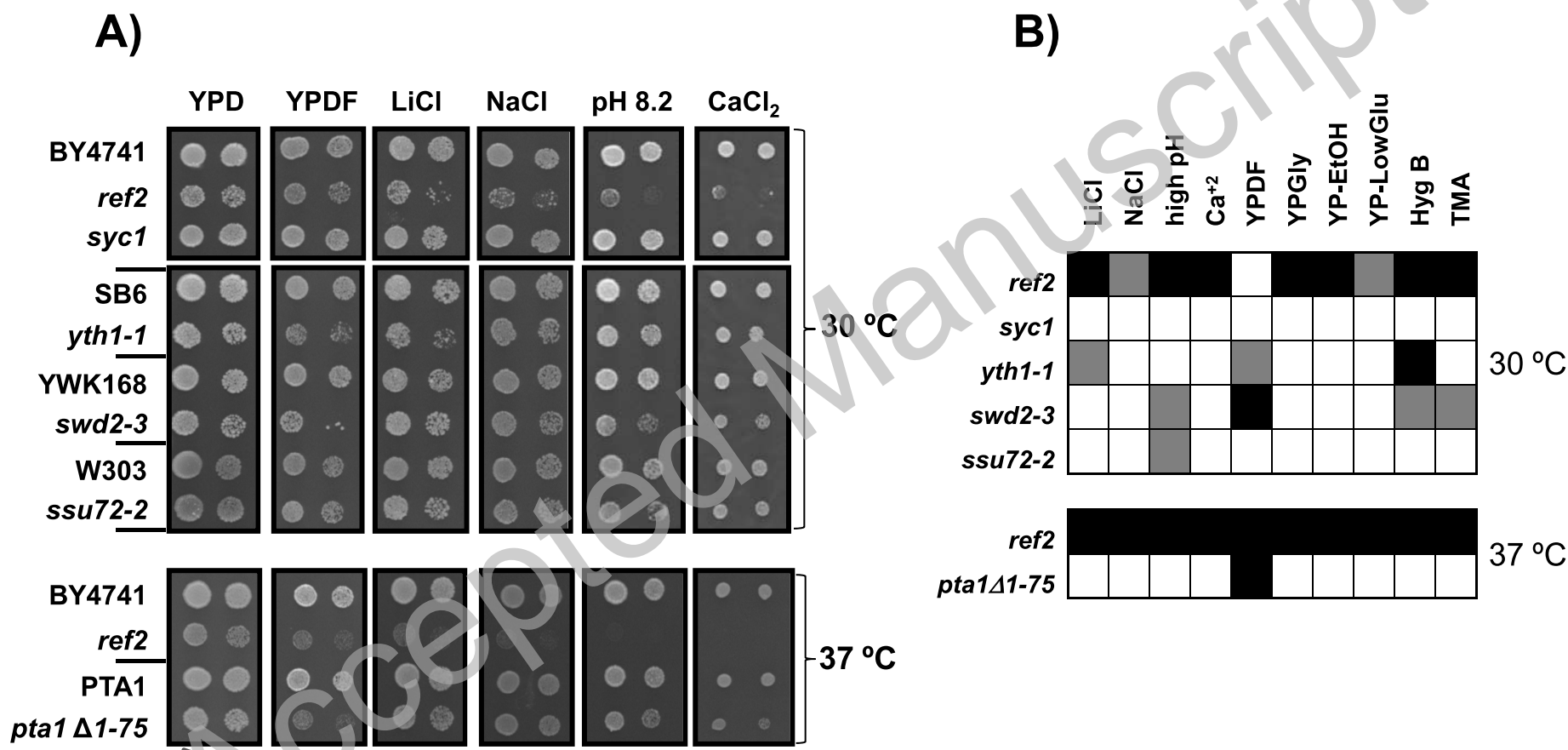


Fig. 7 Ferrer-Dalmau *et al.*

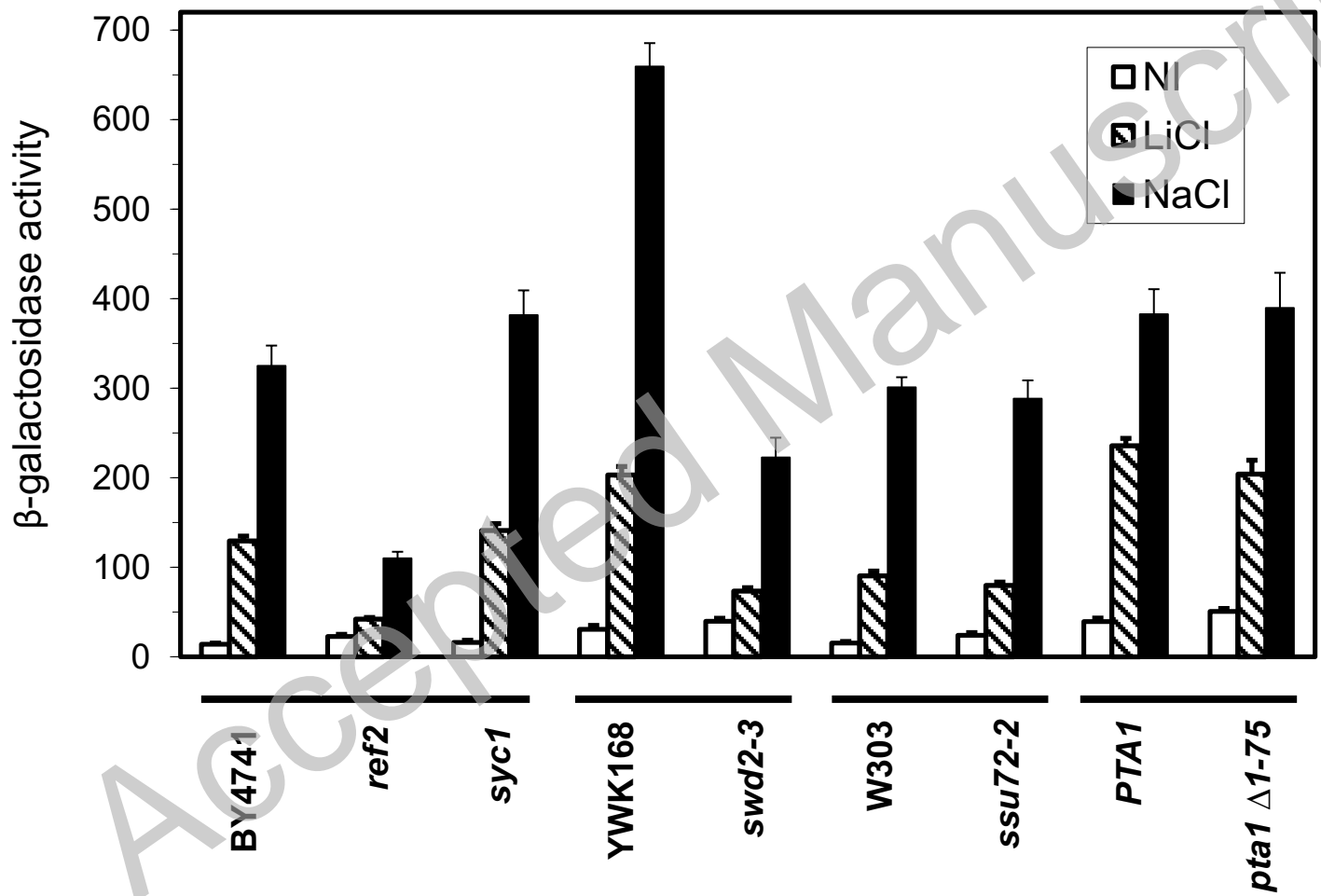


Fig. 8 Ferrer-Dalmau *et al.*