Modulation of Yeast Alkaline Cation Tolerance by Ypi1 Requires Calcineurin

Maribel Marquina,* Asier González,* Lina Barreto,* Samuel Gelis,† Iván Muñoz,*,[1] Amparo Ruiz,*,[2] Mari Carmen Álvarez,† José Ramos,† and Joaquín Ariño*,[3]

*Institut de Biotecnologia i Biomedicina and Departament de Bioquímica i Biologia Molecular, Universitat Autònoma de Barcelona, Bellaterra 08193, Barcelona, Spain, and †Departamento de Microbiología, Universidad de Córdoba, Campus Rabanales, 14071 Córdoba, Spain

ABSTRACT Ypi1 was discovered as an essential protein able to act as a regulatory subunit of the *Saccharomyces cerevisiae* type 1 protein phosphatase Glc7 and play a key role in mitosis. We show here that partial depletion of Ypi1 causes lithium sensitivity and that high levels of this protein confer a lithium-tolerant phenotype to yeast cells. Remarkably, this phenotype was independent of the role of Ypi1 as a Glc7 regulatory subunit. Lithium tolerance in cells overexpressing Ypi1 was caused by a combination of increased efflux of lithium, mediated by augmented expression of the alkaline cation ATPase *ENA1*, and decreased lithium influx through the Trk1,2 high-affinity potassium transporters. Deletion of *CNB1*, encoding the regulatory subunit of the calcineurin phosphatase, blocked Ypi1-induced expression of *ENA1*, normalized Li^+ fluxes, and abolished the Li^+ hypertolerant phenotype of Ypi1-overexpressing cells. These results point to a complex role of Ypi1 on the regulation of cation homeostasis, largely mediated by the calcineurin phosphatase.

MAINTENANCE of alkali cation homeostasis is a key process in most types of cells, including yeasts. The budding yeast *Saccharomyces cerevisiae* is able to grow in the presence of a broad range of external concentrations of K^+ (10 μM–2.5 M) and Na^+ (<1.5 M). Entry of these cations is facilitated by a proton motive force across membranes, generated by the Pma1 H^+-ATPase (Serrano *et al.* 1986), which serves as a source of energy to pump alkali metal cations against their gradients (see Arino *et al.* 2010 for a recent review).

S. cerevisiae is able to concentrate potassium through the high-affinity potassium transporters Trk1 and Trk2, Trk1 being the most functionally relevant. These transporters also allow entry of sodium or even lithium cations. Efflux of excess sodium can be mediated through the Na^+/H^+ Nha1 antiporter, which also transports potassium (Banuelos *et al.* 1998). This mechanism is highly effective in cells growing in an acidic medium. However, *S. cerevisiae* has P-type ATPases (the ENA system), which are able to pump out sodium cations. Usually the *ENA* genes appear in clusters and different strains can have a different number of genes (from 1 to 5). *ENA* genes are normally expressed at very low levels but at least one member of the cluster (*ENA1*) is rapidly activated by salt stress or alkalinization of the medium (see Ruiz and Arino 2007; Rodriguez-Navarro and Benito 2010 for reviews). Besides sodium, the Ena1 ATPase can also transport potassium and lithium cations.

To ensure a correct balance between influx and efflux of cations, their transport across the cell membrane must be regulated. Interestingly, in many cases the regulatory mechanisms involve phospho-dephosphorylation reactions and, therefore, protein kinase and phosphatase activities. For instance, efflux of cations through the *ENA1* ATPase is mainly regulated at the transcriptional level, involving several signaling pathways that allow fine-tuned control of its expression. These pathways include diverse kinases (Hog1, PKA, or Snf1), and phosphatases, such as calcineurin, Ptc1, or Ppz1/Hal3 (Ruiz and Arino 2007). In contrast, Nha1 levels do not significantly vary, although Nha1's regulation also entails protein kinase and phosphatase activities in response to cation stress (Proft and Struhl 2004; Manlandro *et al.* 2005; Kinclova-Zimmermannova and Sychrova 2006).

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[1]Present address: MRC Protein Phosphorylation Unit, College of Life Sciences, University of Dundee, Dundee DD1 5EH, Scotland, UK.
[2]Present address: Department of Genetics and Development, Columbia University, New York, NY 10032.
[3]Corresponding author: Institut de Biotecnologia i Biomedicina, Universitat Autònoma de Barcelona, Cerdanyola del Vallès 08193, Barcelona, Spain. E-mail: Joaquin.Arino@uab.es

There is no experimental evidence for transcriptional regulation of *TRK1* under cation-related stresses. The Hal4/Sat4 and Hal5 protein kinases were found to activate potassium transport by positively modulating Trk1 and Trk2 (Mulet *et al.* 1999), although further work revealed that Hal4 and Hal5 may not be directly required for Trk1 activity, but for stabilizing the transporter at the plasma membrane under low-K^+ conditions (Perez-Valle *et al.* 2007). Trk function is also negatively controlled by the Ppz phosphatases, represented in *S. cerevisiae* by a pair of genes, *PPZ1* and *PPZ2*, the former being the most physiologically relevant in cation homeostasis (Posas *et al.* 1995; Clotet *et al.* 1996). A *ppz1 ppz2* strain shows increased *ENA1* expression but also higher-than-normal potassium uptake (Yenush *et al.* 2002). It has been shown that Ppz1 physically interacts with Trk1. Trk1 is phosphorylated *in vivo*, and its level of phosphorylation increases in *ppz1 ppz2* mutants (Yenush *et al.* 2005), although a direct effect of the phosphatases on the transporter has not been demonstrated. An early report showed that calcineurin is necessary for the transition of the Trk transporter system to the high-affinity potassium state induced by Na^+ stress (Mendoza *et al.* 1994), although the basis for this effect is still unknown. Calcineurin has recently been reported to also be necessary for normal Trk1 activity even under basal conditions (Casado *et al.* 2010).

The gene *GLC7* encodes the catalytic subunit of the essential type 1 protein phosphatase (Clotet *et al.* 1991; Feng *et al.* 1991), an enzyme involved in a myriad of cellular functions. Functional specificity is often gained by interaction of Glc7 with diverse regulatory polypeptides that determine Glc7 intracellular localization and/or substrate recognition. Many of these regulatory proteins have a conserved binding site, with a (R/K)(V/I)X(F/W) consensus sequence, which is necessary for the interaction with an hydrophobic groove in the Glc7 molecule (Bollen *et al.* 2010). Ypi1 is an essential regulatory subunit of Glc7 that plays a role in progression of mitosis; although whether it acts as a positive or negative regulator of the phosphatase, is a matter of controversy (Garcia-Gimeno *et al.* 2003; Pedelini *et al.* 2007; Bharucha *et al.* 2008). Ypi1 has a ^{48}RHNVRW^{53} sequence, which is required for interaction with Glc7 and can be found as a complex with Glc7 and Sds22 (another essential subunit of Glc7) (Pedelini *et al.* 2007). During the course of our initial characterization of this gene product, and due to the essential nature of Ypi1, we performed a number of experiments by overexpressing the protein under different circumstances (Garcia-Gimeno *et al.* 2003). We noticed that cells overexpressing Ypi1 showed increased tolerance to alkali cations, particularly to lithium. Since a role in ion homoeostasis was postulated for Glc7 some years ago as a result of the characterization of the *glc7-109* allele (Williams-Hart *et al.* 2002), we considered the possibility that Ypi1 might be acting on Glc7 in regulating cation tolerance. In this article we report that, although Ypi1 is certainly involved in normal lithium tolerance, to our surprise this phenotype appears not to be mediated by Glc7. We show that this effect is likely caused by a combination of increased lithium efflux through the Ena1 cation ATPase and decreased influx through the Trk1,2 high-affinity potassium uptake system and that these effects require a functional calcineurin signaling pathway.

Materials and Methods

Yeast strains and growth conditions

S. cerevisiae strains used in this work are listed in Table 1. Yeast cells were grown at 28° in YPD medium (10 g/liter yeast extract, 20 g/liter peptone, and 20 g/liter dextrose) or, when carrying plasmids, in synthetic complete medium (Adams *et al.* 1997) containing 2% glucose and lacking the appropriate selection requirements. The BY4741 derivative strain BYT5 is a kind gift from Hana Sychrová (Institute of Physiology, Academy of Sciences of the Czech Republic) and it was constructed by homologous recombination using the Cre-loxP system (Guldener *et al.* 1996). Strain MP002 was generated, transforming JA100 cells with the *cnb1*::*KanMX4* disruption cassette described in Serrano *et al.* (2002). Strains carrying a *HIS3* disruption of the *CNB1* gene (MMR38 to MMR41) were constructed using plasmid pST1.13 as described in Mendoza *et al.* (1994). Strain MMR37-1 (*ena1-5*::*loxP crz1*::*KanMX4*) was constructed by transformation of strain BYT5 with a 3.0-kbp *kanMX* cassette obtained from the deletion collection (EUROSCARF) *crz1* strain by amplification with oligonucleotides 5′_CRZ1_disr (5′-tagcagttgagctccagc-3′) and 3′_CRZ1_disr (5′-gctctagaacagtggctcgtacc-3′).

Plasmids

High-copy expression of *YPI1* and *YPI1^{W53A}* from the *ADH1* promoter was accomplished by transforming cells with plasmids pWS93-*YPI1*, pWS93-*YPI1W53A*, pSK93-*YPI1*, and pSK93-*YPI1W53A* (Garcia-Gimeno *et al.* 2003). The following *LacZ* reporter plasmids, used in the present study, have been described previously. Plasmid pKC201 contains the entire promoter of *ENA1* (Alepuz *et al.* 1997), plasmid pAMS366 contains four copies in tandem of the wild-type calcineurin-dependent response element (CDRE) present in the *GSC2/FKS2* promoter, while pAMS364 contains a mutated version that cannot bind Crz1 (Stathopoulos and Cyert 1997). High-copy expression of *HAL3* was accomplished by transforming cells with the episomal plasmid YEplac195-HAL3 (Munoz *et al.* 2004).

Growth tests

Growth in solid media was assessed by drop tests. Briefly, saturated cultures in synthetic medium lacking uracil or tryptophan, as needed, or in YPD, were diluted until an OD_{660} of 0.05 and 1:5 serial dilutions were prepared. Three microliters from each dilution were spotted on the indicated plates and incubated for the specified time at 28°. Growth in liquid medium in the presence of LiCl was determined as in

Table 1 Yeast strains used in this work

Name	Relevant genotype	Source/reference
CML476	*MAT***a** *ura3-52 leuΔ1 his3Δ200 GAL2* CMVp(tetR`-SSN6)::*LEU2 trp1*::*tTA*	Yen *et al.* (2003)
MMR09-4	CML476 *tetO7:YPI1*	Pedelini *et al.* (2007)
MMR40-2	CML476 *cnb1*::*HIS3*	This work
MMR41-3	MMR09-4 *cnb1*::*HIS3*	This work
DBY746	*MATα ura3*-52 *leu2*-3, 112 *trp*1-Δ239 *his3*-Δ1	D. Botstein
EDN2	DBY746 *ppz1*::*TRP1*	Ruiz *et al.* (2003)
EDN85	DBY746 *ppz1*::*TRP1 ppz2*::*KanMX4*	Ruiz *et al.* (2003)
MAR15	DBY746 *cnb1*::*KanMX4*	Serrano *et al.* (2002)
KT1112	MATa *his3 leu2 ura3-52*	Stuart *et al.* (1994)
KT1623	KT1112 *glc7-127*	Venturi *et al.* (2000)
KT2210	KT1112 *glc7-F256A*	Wu and Tatchell (2001)
KT1935	KT1112 *glc7-109*	Williams-Hart *et al.* (2002)
BY4741	*MAT***a** *his3-1 leu2 met1-5 ura3*	Winzeler *et al.* (1999)
BYT1	BY4741 *trk1*::*loxP*	Petrezselyova *et al.* (2011)
BYT12	BY4741 *trk1*::*loxP trk2*::*loxP*	Petrezselyova *et al.* (2010)
BYT5	BY4741 *ena1-5*::*loxP*	H. Sychrová
BY4741 cnb1	BY4741 *cnb1*::*kanMX*	Winzeler *et al.* (1999)
BY4741 crz1	BY4741 *crz1*::*kanMX*	Winzeler *et al.* (1999)
MMR37-1	BY4741 *ena1-5*::*loxP crz1*::*KanMX*	This work
MMR38-2	BY4741 *cnb1*::*HIS3*	This work
MMR39-3	BY4741 *cnb1*::*HIS3 crz1*::*KanMX*	This work
JA100	*MAT***a** *ura3-52 leu2-3, 112 his4 trp1-1 can-1*^{r}	de Nadal *et al.* (1998)
EDN75	JA100 *ppz1*::*KanMX4*	de Nadal *et al.* (2001)
MP002	JA100 *cnb1*::*KanMX4*	This work
JA104	JA100 *hal3*::*LEU2*	Clotet *et al.* (1999)
ASY832	*TRP1-4xCDRE-lacZ*::*ura3-52*	Kafadar and Cyert (2004)

Posas *et al.* (1995). For testing growth of each mutant under limiting potassium concentrations, cells were grown in YPD medium until saturation. Then, cells were diluted to OD_{600} 0.004 in Translucent K^+-free medium (Navarrete *et al.* 2010) supplemented with different concentrations of KCl and incubated at 28°. The OD_{600} was measured after 16 hr and 24 hr of incubation. The growth percentage value for wild-type strain was determined relative to growth in 50 mM KCl. The same value for BTY1 and BTY12 strains was determined relative to growth in 100 mM KCl.

Li^+ content and Li^+ fluxes assays

To analyze intracellular Li^+, cells were grown overnight in YPD medium containing various LiCl concentrations to an OD_{600} of 0.3. Samples were collected on Millipore filters and rapidly washed with 20 mM $MgCl_2$. The cells were then treated with an acid solution (0.2 M HCl and 10 mM $MgCl_2$) and the cellular extracts were analyzed by atomic emission spectrophotometry (Ramos *et al.* 1990; Ferrer-Dalmau *et al.* 2010).

Li^+ influx was studied in YPD grown cells (OD_{600} of 0.3). At time 0, 100 mM LiCl was added and samples were taken periodically. To determine Li^+ efflux, cells were grown overnight in YPD, adjusted to OD_{600} of 0.3, supplemented with 100 mM LiCl and growth resumed for 1 hr. At time 0, cells were resuspended in lithium-free YPD and aliquots of the culture were withdrawn at various times. In both assays, samples were treated and intracellular Li^+ content determined as described above. Cation values are expressed as nanomoles per milligram of dry weight of cells. The experiments were repeated at least three times.

β-Galactosidase activity assays

The different strains were transformed with the reporter plasmids pKC201, pAMS364, or pAMS366. Usually, yeast cells were grown to saturation in the appropriate drop-out media and then inoculated in YPD at OD_{660} 0.2. Growth was resumed until OD_{660} of 0.5–0.7 and cultures were then distributed into 1.0-ml aliquots and centrifuged for 5 min at 1620 × *g*. For strains carrying the *tetO:YPI1* cassette, YPD cultures (OD_{660} of 0.01) were made of 100 μg/ml doxycycline and growth resumed for 8 h. Forty milliliters of cultures were centrifuged as above. In all cases supernatants were discarded and cultures were resuspended in YPD medium containing 0.2 M LiCl (induced cells) or without the salt (noninduced cells). Cells were grown for 60 min and β-galactosidase activity was assayed as described (Reynolds *et al.* 1997).

Results and Discussion

Ypi1 affects tolerance to lithium cations in a Glc7-independent manner

The protein phosphatase Glc7 has been related to the maintenance of cation homeostasis. As mentioned above, in our initial characterization of Ypi1 functions (Garcia-Gimeno *et al.* 2003) we made the observation that high-copy expression of

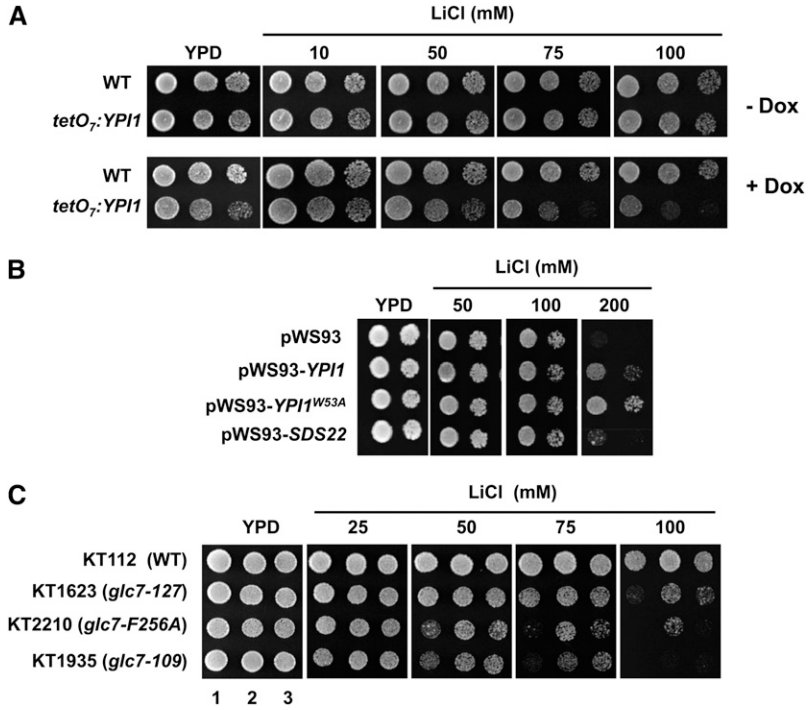

Figure 1 Changes in the amount of Ypi1 alters normal lithium tolerance in a Glc7 phosphatase-independent fashion. (A) Wild-type strain CML476 and its derivative MMR09-4, which carries a chromosomal copy of the Ypi1 gene expressed from a *tetO*-controlled promoter, were grown for 72 hr on YPD in absence (−Dox) or presence of 10 μg/ml doxycycline (+Dox) and the indicated concentrations of LiCl. (B) Wild-type strain BY4741 was transformed with the indicated plasmids and the tolerance to different LiCl concentrations was tested on YPD plates. Growth was monitored after 3 days. (C) Indicated strains derive from wild-type (WT) strain KT112. The relevant *glc7* allele is indicated. Numbers at the bottom denote the plasmid used for transformation. (1) pWS93; (2) pWS93-Ypi1^{W53A}; and (3) pWS93-Ypi1. Tolerance to LiCl was monitored after 3 days of growth.

YPI1 was able to provide a detectable increase in tolerance to alkaline cations, particularly to LiCl, although the basis for this effect was not investigated further. Because Ypi1 is an essential protein, we constructed (Pedelini *et al.* 2007) a strain carrying a conditional allele of *YPI1* in which the protein was expressed from a regulatable *tetO* promoter. To confirm the possible implication of Ypi1 in lithium tolerance, we sought for a specific concentration of doxycycline in which repression of *YPI1* expression could lead to lower-than-normal levels of the protein without excessive effect on cell growth and used this condition to test for altered LiCl sensitivity. As shown in Figure 1A, the presence of 10 μg/ml doxycycline does not affect growth in the absence of LiCl. However, under this condition cells are clearly less tolerant to Li^+, exhibiting very poor growth at 100 mM LiCl, thus confirming that changes in the amount of Ypi1 were able to alter normal Li^+ tolerance. Our attempts to increase the concentration of doxycycline in these long-term experiments to maximize the phenotypic effect were unsuccessful, since even slightly higher amounts of doxycycline already affected growth in the absence of LiCl, thus making comparisons difficult (data not shown). Because of the narrow range of doxycycline that could be used, we were afraid that the presence of the antibiotic may interfere with or mask the phenotypes under study. Consequently, we used a gene overexpression approach to further investigate the role of this regulatory subunit in cation tolerance.

As shown in Figure 1B, whereas overexpression of Ypi1 conferred increased tolerance to LiCl, overexpression of *SDS22*, an essential gene encoding a protein known to associate with Ypi1 and Glc7 (Pedelini *et al.* 2007), did not provide increased LiCl tolerance. Remarkably, overexpression of a version of Ypi1 in which Trp at position 53 had been replaced by an Ala (W53A), was still able to yield Li^+-hypertolerant cells with potency even higher than that conferred by the native protein (Figure 1B). This result was surprising, since mutation of W53 had been reported to abolish the interaction of Ypi1 with Glc7 and, hence, to interfere with the regulatory role of Ypi1 on the phosphatase function (Garcia-Gimeno *et al.* 2003; Hazbun *et al.* 2003; Bharucha *et al.* 2008). To further test whether or not hypertolerance conferred by Ypi1 could be mediated by Glc7 regulation, we overexpressed Ypi1 in strains carrying different *GLC7* alleles, which resulted in hypersensitivity to salts. These alleles were *glc7-127*, which has a weak salt-sensitive phenotype and carries mutations in residues K110 and K112 (Venturi *et al.* 2000); *glc7-109* (K259A and K260A), which is markedly salt sensitive (Venturi *et al.* 2000; Williams-Hart *et al.* 2002); and, more importantly, *glc7*-F256A, which carries a mutation in the hydrophobic groove away from the active site (Wu and Tatchell 2001). This mutation prevents anchoring of RVxF-containing PP1 regulatory subunits to the phosphatase catalytic polypeptide (Bollen *et al.* 2010) and, therefore, should abolish Ypi1 interaction. Interestingly, overexpression of both native Ypi1 and the version containing the W53A mutation were able to confer LiCl tolerance to all *glc7* alleles tested (Figure 1C). Perhaps with the exception of allele *glc7-109*, the effect of the Ypi1^{W53A} version was also more evident than that observed for the wild-type protein. These results indicate that, most likely, the LiCl sensitivity produced by the mutations present in these *glc7* alleles is not caused by the inability to bind Ypi1 and, more importantly, that the Li^+-tolerant phenotype produced by overexpression of Ypi1 is unrelated to the regulation of the Glc7 phosphatase.

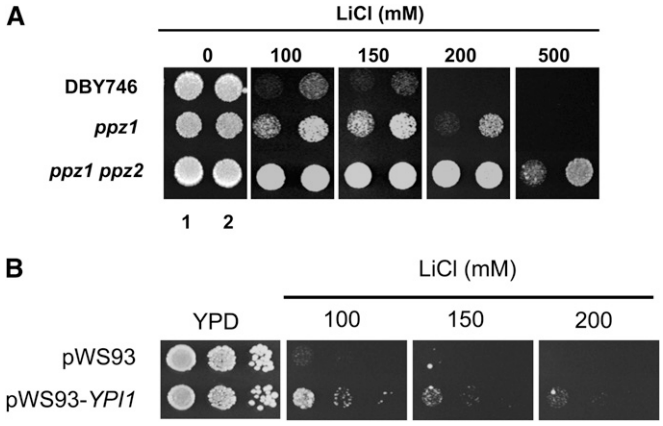

Figure 2 The Ypi1 lithium-tolerant phenotype is independent of the Ppz protein phosphatases. (A) Wild-type strain DBY746 and its isogenic *ppz1* and *ppz1 ppz2* derivatives were transformed with pWS93 (1) or pWS93-Ypi1 (2) and spotted on YPD plates containing the indicated concentrations of LiCl. Growth was monitored after 3 days. (B) Strain JA104 (*hal3*) was transformed with the indicated plasmids and growth in the presence of the indicated concentration of LiCl monitored after 4 days.

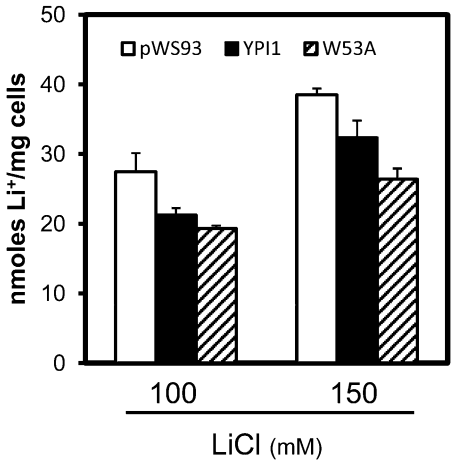

Figure 3 Overexpression of Ypi1 influences intracellular accumulation of lithium. Strain BY4741 was transformed with plasmids pWS93, pWS93-Ypi1 (YPI1), or pWS93-Ypi1^{W53A} (W53A) and grown in the presence of the indicated concentrations of LiCl as indicated in the *Materials and Methods* section. Intracellular Li^+ was measured and is presented as the mean ± SD of five independent experiments.

Ypi1 does not confer lithium tolerance through the Hal3/Ppz system

Ppz1 is a Glc7-related protein phosphatase, and its inhibition results in strong tolerance to alkaline cations (Posas *et al.* 1995; Clotet *et al.* 1996; de Nadal *et al.* 1998). It was reported by two-hybrid analysis that Ypi1 interacts with Ppz1 (Venturi *et al.* 2000) and previous work in our laboratory confirmed a physical interaction between both proteins by GST–pull-down assays (Garcia-Gimeno *et al.* 2003). However, our data indicated that Ppz1 is only modestly inhibited *in vitro* by Ypi1 and that binding (and inhibition) were abolished by mutation of Ypi1 W53. Even though this preliminary information did not support the notion that LiCl hypertolerance conferred by overexpression of Ypi1 could be mediated by Ppz1 inhibition, we expressed Ypi1 in cells lacking Ppz1 or both Ppz1 and Ppz2 phosphatases. As observed in Figure 2A, even in the absence of both phosphatases, Ypi1 was able to increase tolerance to Li^+. In addition, expression of Ypi1 in cells lacking Hal3, in which Ppz1 is abnormally activated, also yielded hypertolerant cells (Figure 2B). Altogether, these observations confirmed that the Ppz enzymes were not involved in the observed Ypi1 phenotype.

Ypi1 controls intracellular lithium accumulation through both Trk1,2- and Ena1-mediated processes

We then sought to understand the basis for the increased tolerance to toxic Li^+ cations provided by high levels of Ypi1. Such phenotype could be the result of lower-than-normal accumulation of the cation under stress conditions or intracellular redistribution of the cation (*i.e.*, by sequestering it into specific compartments). We measured the intracellular concentration of Li^+ in cells grown in the presence of different concentrations of the cation in the medium, and found that cells expressing Ypi1 accumulated less Li^+ than those carrying the empty plasmid (Figure 3). Remarkably, expression of the W53A mutated form of Ypi1 resulted in even lower Li^+ content. This result provided a likely explanation for the hypertolerant phenotype of cells overexpressing Ypi1 and raised the question of whether the decrease in Li^+ content was the result of decreased uptake, increased efflux, or a combination of both events.

To this end, we performed several tests. First, we measured the activation of the *ENA1* promoter by means of a translational *ENA1-lacZ* fusion reporter. *ENA1* encodes a Na^+-ATPase (also able to pump out Li^+), which represents a major component for cation homeostasis and is essentially regulated at the transcriptional level by alkaline cation and high pH stresses (Ruiz and Arino 2007). As shown in Figure 4A, overexpression of Ypi1 results in increased expression from the *ENA1* promoter in wild-type cells exposed to 0.2 M LiCl for 1 hr. The Ppz1 phosphatase has been show to modulate alkaline cation tolerance in part through regulation of *ENA1* expression and, consequently, *ppz1* deletion mutants show higher-than-normal levels of *ENA1* expression both at basal and stress conditions (Posas *et al.* 1995; Ruiz *et al.* 2003). When overexpression was carried out in the *ppz1*Δ strain, we also observed a further increase in *ENA1* expression both in noninduced and Li^+-treated cells. These results suggested that the lower Li^+ content in Ypi1 overexpressing cells could be, at least in part, the result of increased Li^+ efflux mediated by the *ENA1* ATPase, and confirmed this was a Ppz1-independent mechanism. Likewise, when the *ENA1* reporter plasmid was introduced in strain MMR09-4, in which *YPI1* is placed under the control of the *tetO*-regulatable promoter, we observed that repression of *YPI1* expression led to a noticeable reduction in *ENA1* promoter activity (Figure 4B), thus sustaining the notion that Ypi1 activity can control *ENA1* expression. Direct measurement of Li^+ efflux

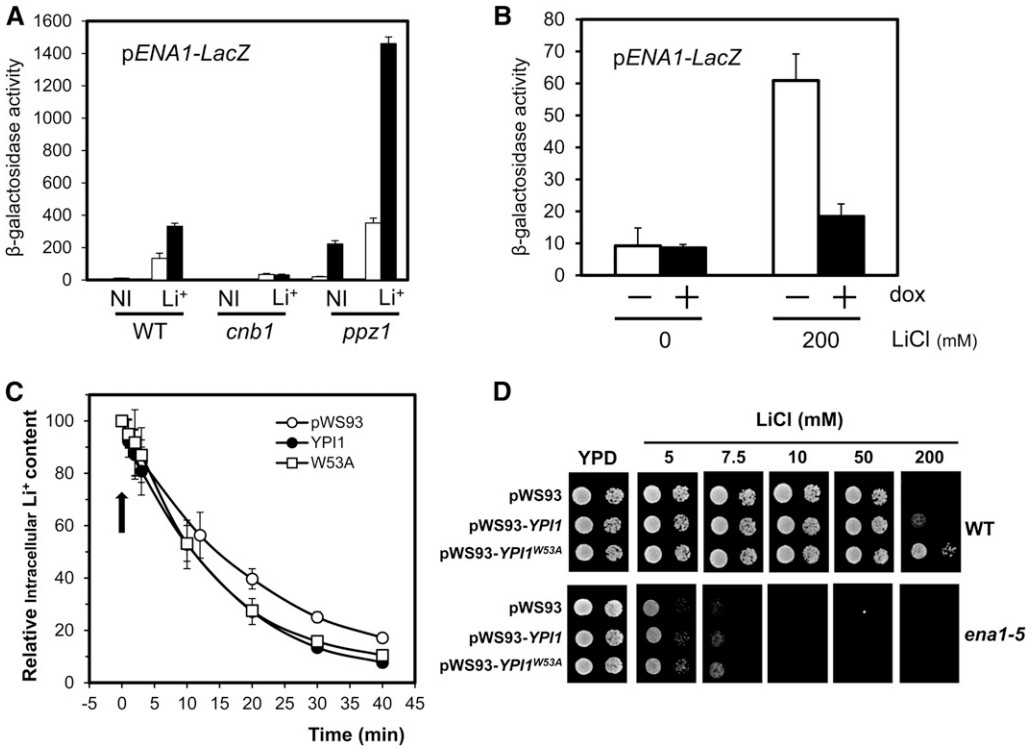

Figure 4 Ypi1 affects *ENA1* expression and lithium efflux. (A) Wild-type strain JA100 (WT) and its derivatives MP002 (*cnb1*) and EDN75 (*ppz1*) were cotransformed with plasmid pSK93 (open bars) or pSK93-Ypi1 (solid bars) and pKC201, which contains the entire promoter of *ENA1*. Cells were treated with 200 mM LiCl for 60 min and β-galactosidase activity was measured. NI, noninduced cells. Data are mean ± SEM of three independent experiments. (B) Strain MMR09-4 (*tetO:YPI1*) was transformed with plasmid pKC201 and grown in YPD medium for 8 hr in the presence of 100 μg/ml of doxycycline (+) or vehicle (−). The medium was made 200 mM LiCl and growth resumed for 1 hr. Cells were collected and assayed for β-galactosidase activity as described. A representative experiment (mean ± SEM from three independent cultures) is shown. (C) BY4741 cells were transformed with the indicated plasmids described above and loaded with lithium for 1 hr as described in the *Materials and Methods* section. At time 0 (marked by an arrow), cells were transferred to a Li^+-free medium, aliquots of the cells were withdrawn periodically, and the amount of intracellular Li^+ was measured. Data are relative to the initial lithium concentration and are mean ± SEM of three independent experiments. (D) Wild-type strain BY4741 (WT) and BYT5 (*ena1-5*) were transformed with the indicated plasmids and spotted on YPD plates containing the indicated concentrations of LiCl. Growth was scored after 3 days.

after 1 hr of loading cells with 100 mM Li^+ showed that yeast overexpressing the native or the W53A version of Ypi1 eliminated Li^+ more effectively than those carrying an empty plasmid (Figure 4C), therefore reinforcing the involvement of the Ena1 ATPase in the Li^+-tolerant phenotype. To further confirm this scenario, we expressed both the native and the W53A version of Ypi1 in cells deleted for the entire *ENA* cluster. As shown in Figure 4D, deletion of the *ENA1-5* cluster substantially compromised the ability of Ypi1 to increase lithium tolerance. However, we could still detect some improvement in tolerance in this highly hypersensitive strain. This would suggest that the effect of overexpression of Ypi1 could not be entirely attributed to increased *ENA1* expression and that other mechanisms must exist.

The effect of Ypi1 overexpression on Li^+ uptake was then examined. As shown in Figure 5A, the presence of Ypi1 decreases the uptake of Li^+ and results in lower-than-normal steady-state levels of the cation 20 min after addition of 100 mM LiCl to the medium. Therefore, the lower Li^+ content (and, hence, the higher tolerance to external Li^+) conferred by Ypi1 would be determined by a combination of a decreased uptake and an increased efflux of the toxic cation. It is known that Li^+ can enter the cell through the high-affinity potassium transport, encoded by genes *TRK1* and *TRK2* (Haro and Rodriguez-Navarro 2003). We expressed both the native Ypi1 and $Ypi1^{W53A}$ versions in cells lacking one (*trk1*) and both (*trk1 trk2*) potassium transporters and tested the tolerance of the resulting strains to LiCl. As observed in Figure 5B, while overexpression of both Ypi1 versions still provides tolerance to *trk1* cells, only a minor effect is observed when overexpressed in the double mutant. These results, together with the influx data, strongly suggest that a decrease in Li^+ influx through the Trk potassium transporters represents a significant component for the enhanced tolerance to LiCl conferred by overexpression of Ypi1. Consistently, when short-term influx of lithium was measured in the double *trk1 trk2* mutant, it was not decreased by overexpression of Ypi1 (Figure 5C). Differences in the accumulation of intracellular Li^+ could be observed at longer sampling times (over 20 min), likely caused by the potent induction of the *ENA1* efflux system described above. It should be noted the higher accumulation of Li^+ in the *trk1 trk2* strain compared with wild type (compare A and C in Figure 5). This is most likely due to ectopic entry of Li^+ cations driven by the hyperpolarization of the cell membrane described to occur in *trk1 trk2* cells, which is responsible for the uptake of toxic alkaline cations (Gomez *et al.* 1996; Madrid *et al.* 1998). This ectopic entry would not be affected by Ypi1 levels.

A relevant question arising from the results above would be whether the effect of overexpression of Ypi1 would similarly affect lithium and potassium entry. To this end, we evaluated the growth of cells overexpressing Ypi1 under

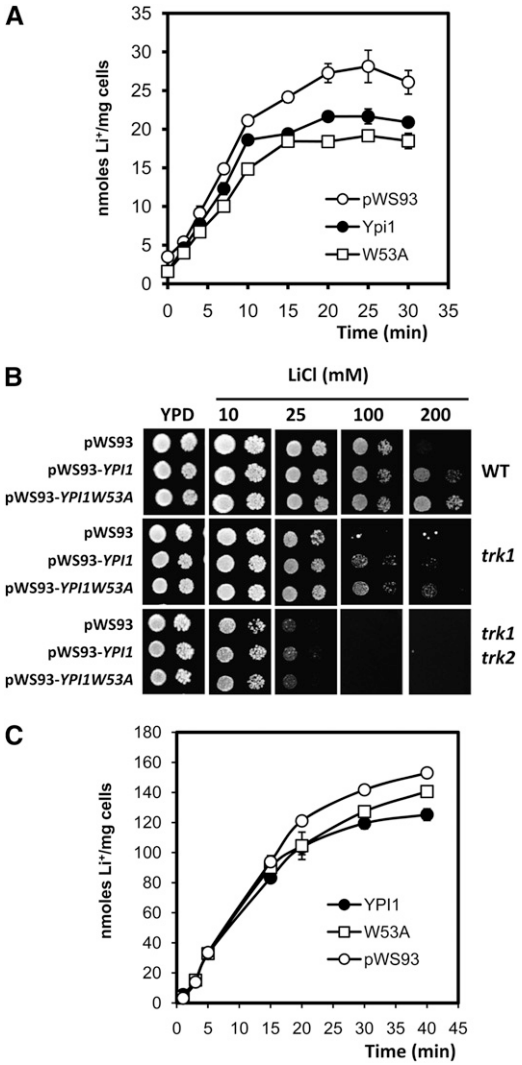

Figure 5 Lithium influx is decreased by overexpression of Ypi1. (A) Cultures of BY4741 cells containing the indicated plasmids were made 100 mM LiCl and aliquots of the cells were withdrawn periodically for intracellular Li^+ measurement. Data are mean ± SEM of three independent experiments. (B) Strain BY4741 and its derivatives BTY1 (*trk1*) and BTY12 (*trk1 trk2*) were transformed with the indicated plasmids and spotted on YPD plates containing the indicated amounts of LiCl. Growth was monitored after 3 days. (C) Strain BYT12 (*trk1 trk2*) was transformed with the indicated plasmids and Li^+ influx measured as in A.

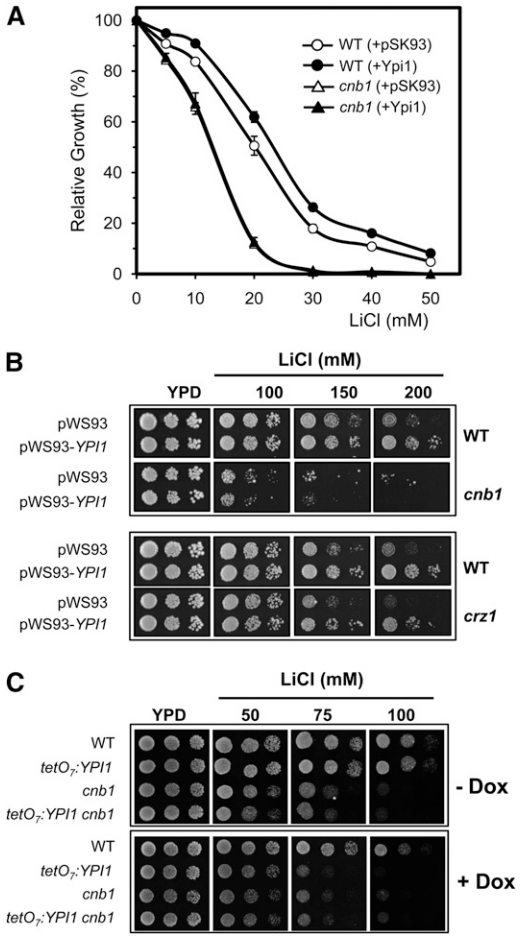

Figure 6 The effect of Ypi1 on Li^+ tolerance is calcineurin dependent. (A) Strain DBY746 and its derivative MAR15 (*cnb1*) were transformed with plasmid pSK93 or the same plasmid expressing Ypi1 and were grown in the presence of the indicated amounts of LiCl for 14 hr. The A_{600} was measured and the results are expressed as percentage of the growth for each strain in the absence of LiCl. Data are mean ± SEM of three independent experiments. (B) Wild-type strain BY4741 and its *cnb1* and *crz1* derivatives were transformed with the indicated plasmids and grown for 3 days in the presence of different concentrations of LiCl. (C) Strains CML476 (WT), MMR09-4 (*tetO7:YPI1*), MMR40-2 (*cnb1*), and MMR41-3 (*tetO7:YPI1 cnb1*) were spotted on YPD plates containing the indicated amounts of LiCl in the absence (−Dox) or the presence (+Dox) of 10 μg/ml doxycycline. Growth was monitored after 72 hr.

limiting potassium concentrations using a newly developed YNB-based, potassium-free medium (Navarrete *et al.* 2010) and calculating the growth ratio at 1 (limiting) and 50 mM (plenty) KCl-containing media (see *Material and Methods*). This ratio was 49.4 ± 0.3 for cells carrying an empty plasmid and 58.8 ± 0.9 for cells overexpressing Ypi1. Therefore, overexpression of Ypi1 does not negatively affect the affinity and/or capacity for potassium transport.

Lithium tolerance conferred by Ypi1 requires a functional calcineurin pathway

During the analysis of the influence of Ypi1 overexpression in *ENA1* expression described above, we observed that lack of *CNB1*, encoding the regulatory subunit of calcineurin, resulted in complete failure to induce expression of the ATPase gene even in cells overexpressing Ypi1 (Figure 4A). Therefore, we considered it necessary to evaluate here the role of the calcineurin signaling pathway. To test this role, we first monitored growth of wild-type and *cnb1* cells transformed with an empty plasmid and with the same plasmid overexpressing Ypi1 in liquid medium containing increasing amounts of LiCl. As can be observed in Figure 6A, the beneficial effect of Ypi1 detected in wild-type cells is completely lost in the hypersensitive *cnb1* strain, thus suggesting that lithium tolerance promoted by Ypi1 could be mediated by calcineurin. This effect was confirmed when

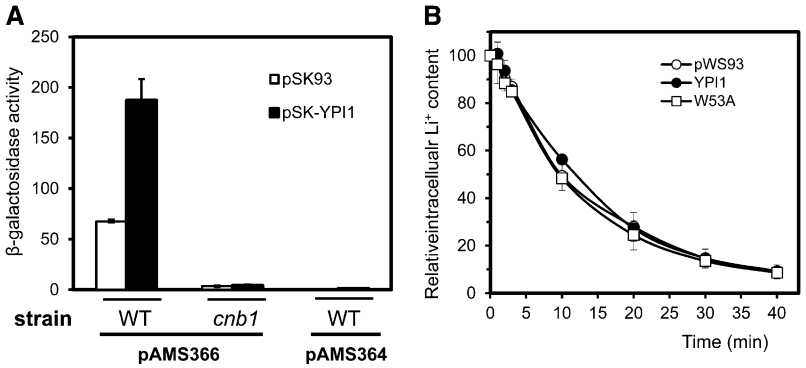

Figure 7 Lack of calcineurin abolishes Ypi1-induced increase in Li^+ efflux. (A) Wild-type strain JA100 (WT) and its derivative MP002 (*cnb1*) were cotransformed with plasmid pSK93 or pSK93-YPI1, together with CDRE-lacZ reporter pAMS366 or pAMS364. Cells were grown to exponential phase and β-galactosidase activity was measured. pAMS364 carries a mutated, nonfunctional version of the CDRE sequence. Data are mean ± SEM of at least three independent experiments. (B) The *cnb1* strain (BY4741 genetic background) was transformed with the indicated plasmids and lithium efflux measured as in Figure 4C.

the *cnb1* mutation was introduced in a different genetic background, and the mutants were grown on YPD agar plates containing various amounts of LiCl (Figure 6B). Deletion of the *CRZ1* gene, encoding the transcription factor downstream of calcineurin (Matheos *et al.* 1997; Stathopoulos and Cyert 1997) reduced, but did not fully eliminate, the effect of overexpression of Ypi1 (Figure 6B). We have also tested the overexpression of Ypi1 in a double *crz1 cnb1* mutant and found that it resulted in a tolerance pattern identical to that of the calcineurin mutant. Similarly, deletion of *CRZ1* in *ena1-5* cells overexpressing Ypi1 had no further effect (data not shown). These results suggest that increased Ypi1 expression would activate calcineurin and increase *ENA1* expression through Crz1 activation. In addition, we constructed the strain MMR41-3, which carries the *cnb1* mutation in the *tetO:YPI1* background. Evaluation of Li^+ tolerance in this strain (Figure 6C) indicates that concurrent lack of calcineurin and depletion of Ypi1 does not show an additive phenotype, further confirming the dependence on calcineurin of the Ypi1 phenotype.

Although *ENA1* expression is certainly known to be controlled by calcineurin (Ruiz and Arino 2007 and references therein), the promoter of the ATPase gene is very complex, integrating different stimuli through various signaling pathways (Marquez and Serrano 1996; Platara *et al.* 2006). Therefore, we sought to test a more specific readout for calcineurin activation upon Ypi1 overexpression. For this purpose, we cotransformed wild-type cells with plasmid pSK93 or pSK93-Ypi1, together with pAMS366 or pAMS364. pAMS366 is a *lacZ*-reporter plasmid that carries a 4× CDRE cluster from the calcineurin-regulatable *FKS2* gene promoter, whereas pAMS364 is the same construct in which the CDRE sequence has been mutagenized to avoid binding of the calcineurin-activated Crz1 transcription factor (Stathopoulos and Cyert 1997). As shown in Figure 7A, overexpression of Ypi1 increases almost threefold the expression from the native CDRE cluster. This effect was calcineurin/Crz1 specific, since it was not observed in cells lacking *CNB1* or carrying the mutated pAMS364 version. An equivalent result was obtained overexpressing Ypi1 in a strain (ASY832) in which the CDRE cluster was integrated in the genome, thus avoiding possible competition between two different plasmids (data not shown).

Finally, we determined Li^+ efflux in cells loaded with LiCl and observed that the increase in Li^+ efflux caused by overexpression of Ypi1 (Figure 4C) was lost in the calcineurin mutant (Figure 7B). Altogether, these results indicate that calcineurin is activated by overexpression of Ypi1, resulting in an increased expression of the *ENA1* ATPase gene, which leads to increased Li^+ efflux and improvement of tolerance. It is remarkable that, in contrast to what can be observed for the *cnb1* mutant, overexpression of Ypi1 is still able to confer some tolerance in cells lacking Crz1, the transcription factor downstream of calcineurin (Figure 6B), thus indicating that Ypi1 may affect, in a calcineurin-dependent fashion, cellular targets relevant for Li^+ tolerance other than the ATPase gene. This would be in agreement with the small, but detectable increase in Li^+ tolerance conferred by Ypi1 in cells lacking the Ena1 efflux system and with the observed contribution of the Trk1,2 potassium influx system. In this regard, it must be noted that an early report by Mendoza *et al.* (1994) identified calcineurin as a necessary element for the transition of the potassium transporter system to the high-affinity state in cells exposed to Na^+ stress, which would help to discriminate K^+ over Na^+, and that more recent evidence from our laboratory indicates that the role of calcineurin is relevant even in the absence of salt stress (Casado *et al.* 2010). As mentioned above, we have observed that overexpression of Ypi1 slightly improves growth under limiting potassium conditions, which indicates that, in contrast to what we observe for lithium influx (Figure 5A), potassium influx would not be decreased. Therefore, the results presented here are consistent with a scenario in which Ypi1 might influence calcineurin function on Trk1,2 cation specificity transport (Figure 8). The possibility of specifically affecting transport of different alkaline cations through Trk1 is not farfetched, since there have been documented mutations in Trk1 that fully block potassium transport without affecting influx of Li^+ (Haro and Rodriguez-Navarro 2003). In any case, these results would imply that, although Ypi1 has been found in the nucleus (Pedelini *et al.* 2007), a fraction of the protein could also be localized in the cytosol.

Although the increase in Li^+ tolerance induced by overexpression of Ypi1 is a quite robust phenotype that we have observed in three different wild-type genetic backgrounds,

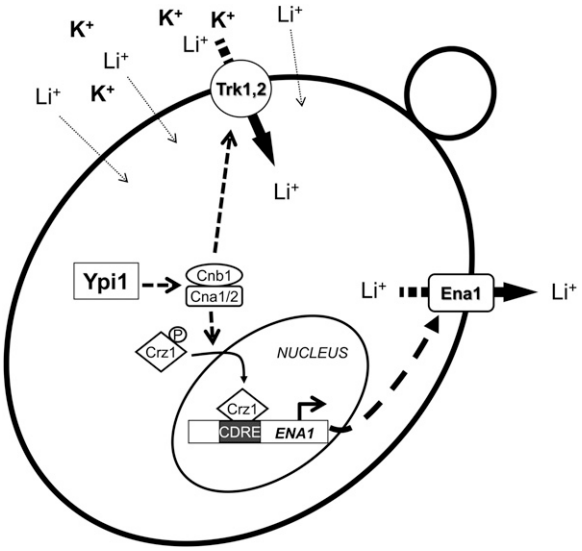

Figure 8 Schematic depiction of the effect of Ypi1 on lithium tolerance. High levels of Ypi1 activate calcineurin, thus triggering activation of *ENA1* expression and increased Li^+ efflux. In parallel, an Ypi1-activated, calcineurin-mediated mechanism, still to be solved, decreases the specificity of the Trk potassium transporter for Li^+ transport, thus avoiding the entry of excessive lithium, in a manner independent on the Ppz1/Hal3 regulatory system (Yenush *et al.* 2002).

the precise mechanism by which Ypi1 might control calcineurin activity toward the Trk1,2 system still remains obscure. We have observed that Ypi1 overexpression does not result in altered tolerance to increased amounts of extracellular calcium (not shown), one of the characteristic phenotypes derived from activation of calcineurin, thus suggesting that not all calcineurin functions are equally activated. Regulatory subunits of Ser/Thr protein phosphatases are thought to be rather selective with regard to the type of catalytic subunit that they modulate, although some crosstalk has been reported in the past (Venturi *et al.* 2000). Therefore, the possibility that Ypi1 could contribute to enhance the activity of calcineurin toward specific substrates (directly or indirectly) should be considered. In this case, the potent phenotype of the Ypi1 version carrying the W53A mutation could be explained because its inability to interact with Glc7 would leave more Ypi1 free to functionally interact with calcineurin.

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