Yeast *ARL1* encodes a regulator of K^+ influx

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Summary

A molecular genetic approach was undertaken in *Saccharomyces cerevisiae* to examine the functions of *ARL1*, encoding a G protein of the Ras superfamily. We show here that *ARL1* is an important component of the control of intracellular K^+. The *arl1* mutant was sensitive to toxic cations, including hygromycin B and other aminoglycoside antibiotics, tetramethylammonium ions, methylammonium ions and protons. The hygromycin-B-sensitive phenotype was suppressed by the inclusion of K^+ and complemented by wild-type *ARL1* and an allele of *ARL1* predicted to be unbound to nucleotide in vivo. The *arl1* mutant strain internalized ~25% more [^{14}C]-methylammonium ion than did the wild type, consistent with hyperpolarization of the plasma membrane. The *arl1* strain took up 30-40% less $^{86}Rb^+$ than did the wild type, showing an inability to regulate K^+ import properly, contributing to membrane hyperpolarity. By contrast, K^+ and H^+ efflux were undisturbed. The loss of *ARL1* had no effect on the steady-state level or the localization of a tagged version of Trk1p. High copy suppressors of the hygromycin-B phenotype included *SAP155*, encoding a protein that interacts with the cell cycle regulator Sit4p, and *HAL4* and *HAL5*, encoding Ser/Thr kinases that regulate the K^+-influx mediators Trk1p and Trk2p. These results are consistent with a model in which *ARL1*, via regulation of *HAL4/HAL5*, governs K^+ homeostasis in cells.

Key words: Guanine nucleotide-binding protein, Ion homeostasis, Membrane potential, *PMA1*, *TRK1*

Introduction

Generation and maintenance of membrane potential are critical for all cells. This property allows for uptake of nutrients, elimination of wastes, generation of cellular energy, and cellular communication in multicellular organisms. This process is highly regulated, allowing cells to respond quickly and effectively to changes in the environment that affect membrane potential. In *Saccharomyces cerevisiae*, membrane potential (negative inside) is determined primarily by efflux of protons via the H^+-ATPase, encoded by the essential *PMA1* gene, and the influx of K^+ via the Trk proteins, encoded by *TRK1* and *TRK2* (reviewed in Gaber, 1992; Serrano, 1996). However, a full understanding of the regulation of this important cellular property is far from complete. Here, we present evidence that the guanine nucleotide-binding protein Arl1p, a member of the Arf-like family of proteins, is an important component in the regulation of K^+ influx, thus affecting membrane potential.

ARL1 is highly conserved over eukaryotic evolution. Yeast, *Arabidopsis thaliana*, *Caenorhabditis elegans*, *Drosophila melanogaster* and mammalian Arl1 proteins are ~60% identical to each other. The *D. melanogaster ARL1* homolog, *arflike*, is essential (Tamkun et al., 1991), although the yeast gene is not (Lee et al., 1997; Rosenwald et al., 2002). We have previously shown that yeast *ARL1* encodes a regulator of membrane traffic (Rosenwald et al., 2002), which has been independently confirmed by others (Bonangelino et al., 2002; Jochum et al., 2002). A role for Arl1 in membrane traffic in mammalian cells has been documented (Icard-Liepkalns et al., 1997; Lowe et al., 1996; Lu et al., 2001; Van Valkenburgh et al., 2001). Furthermore, Arl1 interacts with golgin proteins in both yeast (Panic et al., 2003; Setty et al., 2003) and mammalian cells (Lu and Hong, 2003). However, in this study, we demonstrate an unprecedented role for this guanine nucleotide-binding protein in K^+ homeostasis.

Strains lacking *ARL1* were found to be sensitive to several different cationic translation inhibitors, including hygromycin B. The *arl1* strains also exhibit retarded growth in medium containing methylammonium chloride (MA) or tetramethylammonium chloride (TMA). These phenotypes were suppressed by K^+. In addition, *arl1* strains were sensitive to low pH (pH 3) medium. These results correlated with increased uptake of [^{14}C]-methylammonium ion and decreased $^{86}Rb^+$ uptake by the *arl1* strain. *HAL4* and *HAL5* were isolated as high-copy-number suppressors of the hygromycin-B-sensitive phenotype. These genes encode kinases that function upstream of the K^+ transporters Trk1 and Trk2 (Goossens et al., 2000; Mulet et al., 1999). Loss of *ARL1* did not affect steady-state levels of Trk1 or localization of a tagged version of Trk1, suggesting that, despite Arl1p's documented role as a regulator of membrane traffic, Arl1p acts in a novel manner to control K^+ influx.

Materials and Methods

Yeast strains and growth conditions

The parental strain was PSY316 (*MATα ade2-101 his3-Δ200 leu2-3,112 lys2-801 ura3-52 SSD1*) (Stearns et al., 1990). Replacement of the *ARL1* open reading frame in PSY316 by the *HIS3* gene (*arl1Δ::HIS3*) using a polymerase chain reaction (PCR) disruption

method (Baudin et al., 1993) resulted in strain MA03 (Rosenwald et al., 2002). Strains AM300 and AM310 were created from PSY316 and MA03, respectively, by insertion of a fragment containing the 3′ end of *TRK1* without a stop codon fused to 13 Myc epitopes (Longtine et al., 1998). This fragment was amplified from a template on plasmid pR341 using oligonucleotides GT105* and GT106* (Table 1). Correct insertion of the fragment at the *TRK1* locus was confirmed by PCR using primers GT105, which binds the 3′ end of the *TRK1* open-reading frame, and GT128, which binds to a region 3′ of the *TRK1* open reading frame beyond the insertion. If the insertion is correct, a band of 2.7 kb is amplified. In addition, GT51 (which binds to the KanMX cassette) was used with GT128. These primers, if the insertion is correct, amplify a band of 1 kb.

The homozygous diploid deletion strains shown in Fig. 4 [in the BY4743 background: *MATα/a his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 LYS2/lys2Δ0 MET15/met15Δ0 ura3Δ0/ura3Δ0*, deletions created by replacement of the open reading frame with a *KanMX* cassette (Winzeler et al., 1999)] were from the Deletion Consortium Collection through Research Genetics (Huntsville, AL) or OpenBiosystems (Huntsville, AL).

Strains were routinely grown on YPAD medium or SD medium (Adams et al., 1997; Sherman et al., 1974) at 30°C. In one experiment, galactose (Gal) replaced dextrose in YPAD. SD medium with appropriate supplements to cover auxotrophies was used to confirm genetic markers and for selection. Antibiotics [cycloheximide, hygromycin B, paromomycin, anisomycin (all Sigma Chemical, St Louis, MO) or geneticin (InVitrogen, Rockville, MD)], MA (Sigma) and TMA (Sigma), as well as other cations (as chloride salts; Sigma), were added to YPAD at the concentrations given in the figure and table legends. Antibiotics were added to media after autoclaving. Buffered medium was made as described (Tanida et al., 1995; Withee et al., 1998). Briefly, 0.1 M succinic acid at either pH 3.0 or pH 6.0 was mixed with an equal volume of 2× YPAD, the pH was adjusted with concentrated NaOH to a final pH of 3.0 or 6.0, respectively, then the medium was filter sterilized. Sensitivity to MA (maximum final concentration of 200 mM) was tested in minimal glutamate medium (Soupene et al., 2001).

Plasmids and transformations

The plasmids used in this study are listed in Tables 2 and 3. Oligonucleotides (Table 1) were obtained from InVitrogen (Rockville, MD). The site-directed mutant alleles of *ARL1* in YEp352 (2 micron origin; *URA3*) were described previously (Rosenwald et al., 2002). The high-copy-number suppressor screen was performed using a genomic library (Nasmyth and Reed, 1980) (American Type Culture Collection, Manassas, VA). Library plasmids able to suppress the hygromycin-B-sensitive phenotype of the *arl1* strain are detailed in Table 3.

Several subclones were constructed. Restriction enzymes were either from InVitrogen or New England Biolabs (Beverly, MA). First, plasmid pDH53, containing *MIG1* and YGL034c, was digested with *Eag*I, which cuts once in the insert and once in the vector, removing a 2.2 kb piece including the promoter and the first 50 bp of the YGL034c coding sequence. This was religated to create pDH100. Second, *FUN21* was subcloned by PCR using GT74 and GT75 using pDH15 as template. *Sph*I restriction sites built into the oligonucleotides permitted subcloning to YEp351 at the *Sph*I site (Hill et al., 1986) to make pDH21-18. Third, pMXL244 (vector YEp352), containing the *ATC1* gene, was originally isolated from a library as a high-copy-number suppressor of the temperature-sensitive phenotype of strain AR101 (*arl1Δ::HIS3 ssd1-100*) and includes 737 bp upstream and 802 bp downstream of the *ATC1* open reading frame (Rosenwald et al., 2002).

Yeast transformations were performed using the procedure of Ito et al. (Ito et al., 1983). Plasmids were retrieved from yeast using the procedure of Hoffman and Winston (Hoffman and Winston, 1987). Plasmids were electroporated into electrocompetent *Escherichia coli* (DH10B) following the manufacturer's instructions (InVitrogen). Isolation of DNA from *E. coli* was performed either with Wizard (Promega, Madison, WI) or Qiagen (Valencia, CA) kits.

Library transformants were selected on minimal medium lacking leucine, then replica-printed onto YPAD with 0.1 mg ml^{-1} hygromycin B. Plasmids were isolated from transformants that grew on hygromycin B, transformed into *E. coli*, purified and then analysed by restriction digestion with *Eco*RI, *Hind*III and *Bgl*II. Plasmids were retransformed into MA03 to confirm the suppressing function was on the plasmid. Subclones of the library inserts were tested the same way, except for pERG20, in which *ERG20* is under the control of the inducible *GAL1/10* promoter. Strain MA03 containing pERG20 was grown on YPAGal and YPAGal with 0.1 mg ml^{-1} hygromycin B. Control experiments demonstrated that MA03 transformed with a construct containing *ARL1* under the control of the *GAL1/10* promoter, plasmid pARY1-8, complemented the phenotype on YPAGal with 0.1 mg ml^{-1} hygromycin B.

Miscellaneous

Sequencing of library plasmids was performed with oligonucleotides MP10 and MP11 elongated with Big Dye 3 reagents (Applied Biosystems, Foster City, CA) by PCR. The PCR products were purified on Sephadex G-50 spin columns (Princeton Separations, Princeton, NJ). An Applied Biosystems 377 sequencer was used.

Western blotting for Arl1p using a polyclonal anti-peptide antibody was performed as described (Rosenwald et al., 2002). Trk1-Myc was detected using a commercially available anti-c-Myc antibody (9E10; Roche, Indianapolis, IN). Anti-Vph1p and anti-Pgk1p antibodies were from Molecular Probes (Eugene, OR). Anti-Pma1p antibody was the kind gift of C. Slayman (Yale University). Horseradish-peroxidase-linked secondary antibodies were from Amersham Biosciences (Piscataway, NJ). Detection was performed using the enhanced chemiluminescence (Amersham Biosciences).

Subcellular fractionation was performed essentially as described (Gaynor and Emr, 1997). Briefly, cells were converted to spheroplasts by digestion with zymolyase (25 μg ml^{-1} in 25 mM Tris-HCl, pH 7.5, 1 M sorbitol, 20 mM NaN_3, 20 mM NaF, 10 mM dithiothreitol) for 30-45 minutes at 30°C. Spheroplasts were then lysed by resuspension in a hypo-osmotic buffer (10 mM HEPES-KOH, pH 6.8, 0.2 M sorbitol, 50 mM potassium acetate, 2 mM EDTA, 1 mM dithiothreitol,

Table 1. Oligonucleotides used in this study

GT51	caccactgcgatccccgg
GT74	gcgcgcatgctttcatcaacttttagct
GT75	gcgcgcatgcactaatacttttgacgatc
GT105	gtgaaacatcgttggggagctcttaagcgtaagacc
GT105*	gtgaaacatcgttggggagctcttaagcgtaagaccacacattcccgaaatcctaaaaggagcagcacaacgctccggatccccgggttaattaa
GT106*	aaaatccttttaatgcttaattaccttcttaaatattgagtacgaaaacctatttctaaagaatgagtatatatggaattcgagctcgtttaaac
GT128	ggcccgggatcccggttgtctttaagggaccg
MP10	cttggagccactatcgac
MP11	ccgcacctgtggcgccg

Table 2. Plasmids used in this study

NAME (marker)	INSERT	Source/reference
YEp351 (*LEU2*)	–	Hill et al., 1986
YEp352 (*URA3*)	–	Hill et al., 1986
pARY1-3	*ARL1* in YEp352	Rosenwald et al., 2002
pHV684	*ARL1[G2A]* in YEp352	Rosenwald et al., 2002
pARY1-30	*ARL1[T32N]* in YEp352	Rosenwald et al., 2002
pHV686	*ARL1[Q72L]* in YEp352	Rosenwald et al., 2002
pARY1-32	*ARL1[N127I]* in YEp352	Rosenwald et al., 2002
pARY1-31	*ARL1[D130N]* in YEp352	Rosenwald et al., 2002
pARY1-8	*GAL1/10:ARL1* in pBM272	This study
pMXL-244	*ATC1* in YEp352	Rosenwald et al., 2002
pCAtrB-2	activated calcineurin in YEp352	Mendoza et al., 1996
p*SNC1*	*SNC1* in pRS425	Peter Novick*
p*SNC2*	*SNC2* in pRS426	Peter Novick*
pM73	*HAL4* in YEp351	Mulet et al., 1999
pM89	*HAL5* in YEp351	Mulet et al., 1999
pCK100	*CKI1* in YEp351	George Carman†
pERG20	*GAL1/10:ERG20* in pRS316	Anita Hopper‡
CB2819	*SAP185* in YEp24	Luke et al., 1996
CB2606	*SAP190* in YEp24	Luke et al., 1996
CB2925	*SAP4* in YEp24	Luke et al., 1996
CB2643	*SAP155* in YEp24	Luke et al., 1996
pRG296	*TRK1* in pGN621	Gaber et al., 1988
pDH8	from YEp13 library containing YJL193w	This study
pDH15	from YEp13 library containing *FUN21*	This study
pDH18	from YEp13 library containing *ARL1*	This study
pDH32	from YEp13 library containing *SAP155*	This study
pDH34	from YEp13 library containing *CKI1*	This study
pDH39	from YEp13 library containing *MIG1*	This study
pDH43	from YEp13 library containing *CKI1*	This study
pDH48	from YEp13 library containing *ATC1*	This study
pDH50	from YEp13 library containing *HAL5*	This study
pDH53	from YEp13 library containing *MIG1*	This study
pDH100	*MIG1* in YEp13 (from pDH53)	This study
pDH21-18	*FUN21* in YEp351	This study
pRR193	from YEp13 library containing YJL193w	This study
pR341	source of *myc-KanMX* cassette	Longtine et al., 1998

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containing the protease inhibitors phenylmethylsulfonyl fluoride [20 μg ml^{-1}], antipain [5 μg ml^{-1}], leupeptin [0.5 μg ml^{-1}], pepstatin [0.7 μg ml^{-1}] and α_2-macroglobulin [10 μg ml^{-1}]) and disrupted using a glass tissue homogenizer. After clearing the lysates of unlysed cells and cell debris by low-speed centrifugation, the lysates were then separated by differential centrifugation. For supernatant samples, proteins were first concentrated by precipitation with trichloroacetic acid (Peterson, 1977) before gel electrophoresis. Proteins were identified in each fraction by western blot analysis.

Uptake of [^{14}C]-MA (Amersham Biosciences) was performed essentially as described (Navarre and Goffeau, 2000). Briefly, cells were grown in SD medium with the necessary supplements to cover auxotrophies. Log-phase cells were washed twice with and resuspended in water to give an OD_{600} value of 20 and stored on ice until ready for use. Cells were then diluted 1:1 with 2× reaction buffer for a final concentration of 10 mM 2-[N-morpholino]ethanesulfonic acid (MES), pH 6.0 (adjusted with NaOH), containing 50 mM glucose. The reaction mixture was incubated for 5 minutes at room temperature, then the uptake reaction was initiated by the addition of 2.5 μCi ml^{-1} [^{14}C]-MA (2 mM final concentration). 100 μl aliquots were removed at intervals up to 60 minutes and diluted into 10 ml of ice-cold 20 mM $MgCl_2$. Cells containing [^{14}C]-MA were collected by rapid filtration onto nitrocellulose filters (Schleicher and Scheull, Keene, NH) followed by three 10 ml washes with ice-cold 20 mM $MgCl_2$.

Uptake of $^{86}Rb^+$ (Perkin-Elmer, Boston, MA) was adapted from Mulet et al. (Mulet et al., 1999). Cells were grown in SD medium with the necessary supplements (including 200 mM KCl for experiments in which *trk1* cells were examined). Log-phase cells were washed twice with and then resuspended in water to give an OD_{600} value of 20 and incubated on ice until ready for use. Cells were then diluted 1:1 with 2× reaction buffer for a final concentration of 50 mM succinic acid pH 5.5 (adjusted with Trizma base) containing 4% glucose and incubated for 5 minutes at room temperature. ^{86}RbCl (0.22 μCi ml^{-1}, 0.2 mM final concentration) was added from a concentrated stock and cells were incubated at room temperature for up to 45 minutes. Cells were collected and washed as above for MA uptake. $^{86}Rb^+$ efflux was accomplished by loading cells with ^{86}RbCl as above, then washing once with 1× reaction buffer. Cells were then collected and washed as above.

Proton efflux was measured as described (Perlin et al., 1988). Briefly, cells were grown to mid-log phase (OD_{600} 0.8) in YPAD. Cells diluted to give an OD_{600} value of 40 were washed three times with distilled water, then stored on ice for 1-3 hours. At the end of this period, cells were pelleted by centrifugation and resuspended in 50 ml 25 mM KCl or distilled in water to give an OD_{600} value of 0.8. A pH electrode was suspended in the cell mixture, which was subjected to constant stirring. The pH was measured until a stable baseline was observed, then glucose was added from a 100% stock in water to a final concentration of 2%. The pH was read every 15 seconds until a new steady state was reached, usually within 20-25 minutes.

Table 3. Plasmids recovered from the high copy suppressor screen

Chromosome (coordinates)	Number of isolates	Hygromycin B suppression	Open-reading frames in insert*	Proposed function of protein encoded by the suppressing gene
(empty vector)	–	–	–	–
II (567382-574763)	7	++++	***ARL1***, *UBS1*, *TYR1*, *POP7*, YBR168w	G protein of the Arf-like family
X (103703-109978)	1	++++	*ERG20*, *QCR8*, ***HAL5***	Ser/Thr kinase regulating K^+ transporters, Trk1 and Trk2
VI (228886-239723)	1	+++	YFR038w, YFR039c, ***SAP155***, *ERJ5*	Regulator of Sit4p phosphatase
I (84115- 90958)	1	++	***FUN21***, *SNC1*	Function unknown now
VII (430112-435243)	9+1§	++	***MIG1***, YGL034c**	Transcriptional repressor of the *ENA1* gene, encoding the Na^+/Li^+ efflux pump
X (67860-73441)	1¶	++	*ELO1*, YJL195c**, *CDC6*, **YJL193w**	Shows 31% identity (50% similarity) to Sly41p, a triose phosphate transporter
IV (829075-835598)	1†	++	***ATC1***, YDR185c, YDR186c	High copy suppressor of the Li^+-sensitive phenotype of calcineurin mutants
XII overlapping region‡	2	++	YLR132C, ***CKI1***	Choline kinase, catalyzes the committed step in phosphatidylcholine biosynthesis via the Kennedy pathway

MA03 was transformed with the high copy number plasmid library as described in Materials and Methods. After confirming that the suppressing function was contained on the plasmid, the plasmids were analyzed by restriction digestion with *Eco*RI, *Hind*III and *Bgl*II, and sequenced. The nucleotide coordinates of each distinct plasmid obtained from the screen is shown, as well as the level of ability to suppress the hygromycin-B-sensitive phenotype. Individual open-reading frames were subcloned and tested as described in Results and the 'bold' gene or open-reading frame was able to suppress alone.

*Open reading frames according to the *Saccharomyces* Genome Database (http://www.yeastgenome.org).

†We also obtained a second clone nearly identical to this one, but one from which the region encoding the C-terminal 20% of *ATC1* (35 amino acids) was replaced with sequences from the vector encoding 39 amino acids.

‡Overlapping fragments: pDH43 contained nucleotides 405602-411144 and pDH34 contained nucleotides 406804-411134 . Both contained YLR132C and *CKI1*.

§A tenth isolate, pDH39, contained a slightly larger fragment (chromosome VII, nucleotides 429435-435243), but nevertheless included both *MIG1* and YGL034c.

¶We obtained a second clone, pRR193, that overlapped with this one and contained YJL193w and YJL192c (chromosome X, nucleotides 69942-74433).

**These have been denoted as 'dubious ORFs' according to the *Saccharomyces* Genome Database.

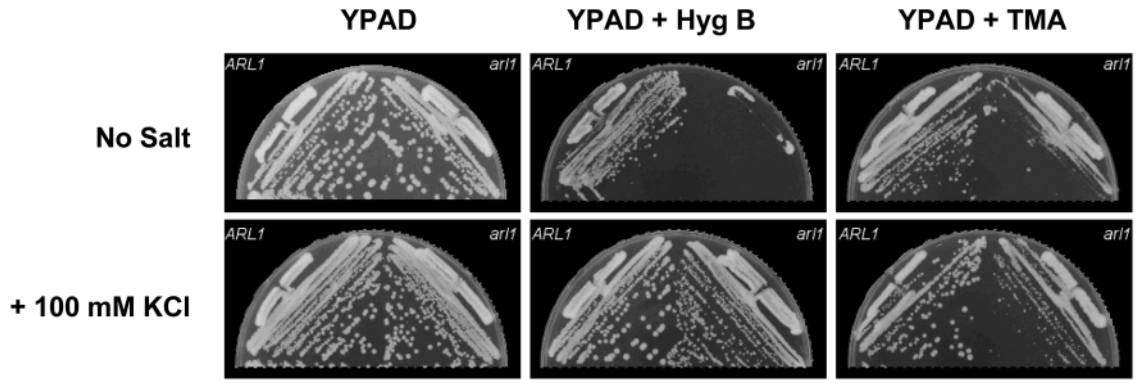

Fig. 1. Strains lacking *ARL1* are sensitive to hygromycin B and TMA. The parental strains PSY316 (*ARL1*; left) and MA03 (*arl1*; right), were struck onto YPAD medium (Adams et al., 1997; Sherman et al., 1974) containing no additions (left), 0.1 mg ml^{-1} hygromycin B (middle) or 0.25 M TMA (right), without other additions (top) or with the addition of 100 mM KCl (bottom). The plates were incubated for 3 days at 30°C and photographed.

Results

arl1 mutants are sensitive to hygromycin B and related compounds

An *arl1* strain, MA03, was found to be extremely sensitive to hygromycin B (Fig. 1), a cationic aminoglycoside that inhibits translation (Brodersen et al., 2000). Growth of MA03 on media containing the related compounds geneticin (G418, added to YPAD medium at a concentration of 25 μg ml^{-1}) and paromomycin (0.25 μg ml^{-1}) was also strongly inhibited. The *arl1* strain was also sensitive to a structurally unrelated cationic translation inhibitor anisomycin (20 μg ml^{-1}). However, addition of cycloheximide (0.3 μg ml^{-1}), which is not a cation, inhibited growth of parental and *arl1* strains to a similar extent (data not shown), suggesting that inhibition of translation per se was not the cause of the phenotype observed.

Eight independent *arl1* isolates derived from the same parent exhibited the same hygromycin B and anisomycin phenotypes. The homozygous diploid *arl1::KanMX* deletion strain from the

Deletion Collection was also sensitive to hygromycin B (see below), whereas most of the other strains in the collection were not (G. L. Fell and A. G. Rosenwald, unpublished). Upon backcross of an *arl1::HIS3* strain to an *ARL1* strain of the opposite mating type and sporulation of the resulting diploid, the His^+ phenotype segregated 2:2 and all His^+ spores were sensitive to hygromycin B. Transformation of *arl1* strains with either a multicopy plasmid (Fig. 2) or a low-copy-number plasmid (data not shown) containing wild-type *ARL1* complemented the hygromycin-B-sensitive phenotype. Hygromycin-B sensitivity is therefore tightly linked to the loss of *ARL1*.

Site-directed mutant alleles of *ARL1* and the hygromycin B phenotype

Because *ARL1* encodes a protein that binds guanine nucleotides (Lee et al., 1997), we tested the hypothesis that the nucleotide-binding state of Arl1 protein was important for its function with respect to the hygromycin-B-sensitive phenotype. The *arl1* strain was transformed with high-copy-number plasmids, each containing a different site-directed mutant allele of *ARL1*, including those predicted to alter nucleotide binding and hydrolysis. The wild-type allele complemented the phenotype (Fig. 2). The myristoylation mutant *ARL1[G2A]* was unable to complement this phenotype, demonstrating that this modification was important for activity. The alleles encoding mutants predicted to have high exchange rates for or to bind only GDP, *ARL1[N127I]* and *ARL1[T32N]*, respectively, did not complement this phenotype. Interestingly, the mutant predicted to be unable to hydrolyse GTP, *ARL1[Q72L]*, complemented the phenotype weakly (Fig. 2). Surprisingly, only the allele encoding a protein predicted to be unbound to nucleotide, *ARL1[D130N]*, complemented as well as the wild type. This mutant, by analogy to Ras (Powers et al., 1991; Powers et al., 1989), Ypt1 (Jones et al., 1995) and EF-Tu (Hwang and Miller, 1987), is predicted to bind xanthine nucleotides preferentially, and so to be 'empty' in vivo. As a control, western-blot analysis of lysates with an anti-Arl1-peptide antibody (Rosenwald et al., 2002) demonstrated that, in all strains transformed with an *ARL1*-containing plasmid, a ~20 kDa band was observed. This band was not observed in cells transformed with empty vector (data not shown). Lack of complementation by these alleles cannot therefore be ascribed to lack of expression. Taken together, these results suggest that the relevant conformation of Arl1p for complementation of this phenotype might be the nucleotide-free form rather than the GTP-bound form.

arl1 mutants show increased uptake of a toxic cation

Mutations in several genes have been shown to cause sensitivity to hygromycin B and other toxic cations. These include the genes for calcineurin (Withee et al., 1998), the K^+ influx transporters Trk1p and Trk2p (Madrid et al., 1998), and two kinases that regulate these transporters, Hal4p and Hal5p (Mulet et al., 1999), all of which are thought to regulate membrane potential. To determine whether the loss of *ARL1* caused a phenotype consistent with inability to regulate membrane potential, we performed several additional experiments.

First, inclusion of K^+ in the growth medium suppressed the hygromycin-B-sensitive phenotype of the *arl1* mutant very well (Fig. 1). Second, as described above, *arl1* strains were sensitive to a range of other cationic translation inhibitors in addition to hygromycin B. Additionally, the *arl1* strain was sensitive to several other toxic cations that do not inhibit translation, including TMA (Fig. 1). Sensitivity to TMA was also suppressed by K^+ (Fig. 1). The *arl1* strain was also sensitive to protons. Although *ARL1* and *arl1* strains grew at the same rate in medium buffered to pH 6.0, the *arl1* strain grew substantially more slowly in medium buffered to pH 3.0 than the parental wild-type strain (data not shown). Similarly, the *arl1* strain grew substantially more slowly in medium containing 25 mM MA (data not shown). Thus, the *arl1* mutant showed a general sensitivity to toxic cations and not to cationic translation inhibitors specifically. Third, because the Ca^{2+}/calmodulin-dependent protein phosphatase calcineurin is a major regulator of cellular responses to environmental ionic change, we transformed the *arl1* mutant strain with an activated calcineurin construct, pCAtrB-2, containing full-length *CNB1* and truncated *CNA1*, missing the calmodulin-binding and autoinhibitory domains (Mendoza et al., 1996). This construct

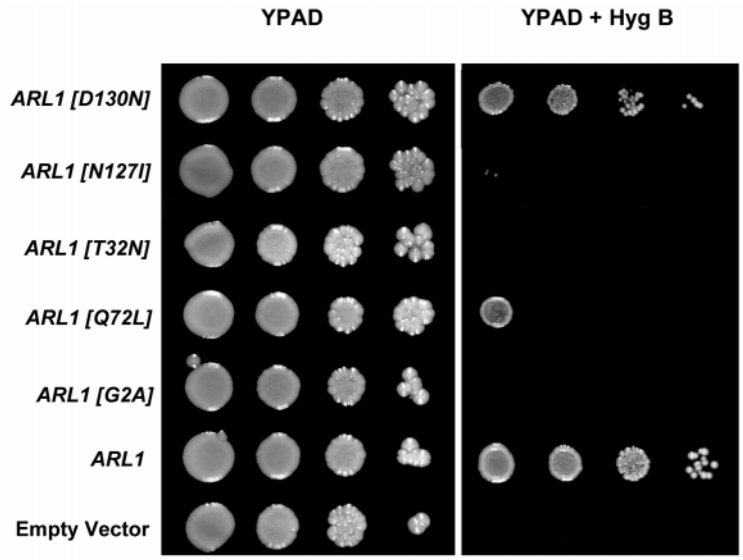

Fig. 2. Alleles of *ARL1* and their ability to complement the hygromycin-B-sensitive phenotype of *arl1* strains. The *arl1* strain MA03 was transformed with the constructs listed, then grown overnight in minimal medium lacking uracil. The overnight cultures were subjected to tenfold serial dilutions in water and then spotted onto YPAD plates without (left) or with (right) hygromycin B (0.1 mg ml^{-1}) using a replicator tool. The plates were incubated at 30°C for 3 days and photographed.

suppressed both the hygromycin-B-sensitive and the TMA-sensitive phenotypes of the *arl1* mutant (data not shown).

Finally, the *ARL1* and *arl1* strains were incubated with a radioactive version of one of the toxic cations, [^{14}C]-MA. This cation has been used as a tracer by others to demonstrate hyperpolarity in yeast (Madrid et al., 1998; Mulet et al., 1999; Navarre and Goffeau, 2000). The *arl1* mutant strain took up 25% more than the wild type after 60 minutes (Fig. 3). In addition, inclusion of K^+ in the buffer decreased the amount of uptake in both wild-type and *arl1* strains, but to a greater extent in the *arl1* strain, bringing the amount of uptake observed within the amounts seen in wild-type cells (data not shown). The observed increase in [^{14}C]-MA uptake upon deletion of *ARL1* was of the same order of magnitude relative to the wild type as has been observed by others for genes with established roles in ion homeostasis (Madrid et al., 1998; Mulet et al., 1999). In summary, because the *arl1* strain was sensitive to a range of structurally unrelated cations and the sensitivity phenotypes could be suppressed by inclusion of K^+ or calcineurin, these results are consistent with the hypothesis that cells lacking *ARL1* are hyperpolarized, which in turn leads to increased uptake of toxic cations.

High-copy-number suppressors of the *arl1* hygromycin-B-sensitive phenotype

To identify genes that act downstream of *ARL1*, we performed a high-copy-number suppressor screen by transforming a genomic library (Nasmyth and Reed, 1980) into the *arl1* strain. We obtained several different genes, all of which also suppressed the TMA-sensitive phenotype (Table 3; data not shown). Seven identical plasmids, exemplified by pDH18, suppressed very well and contained a portion of chromosome II that included *ARL1*. Because multiple *ARL1* isolates were found, sufficient numbers of transformants were obtained to cover the genome. We also identified three genes that appeared to function downstream of *ARL1*.

Plasmid pDH50 contained three genes: *ERG20*, *QCR8* and *HAL5*. *ERG20* encodes farnesyl pyrophosphate synthetase (Anderson et al., 1989; Szkopinska et al., 2000) and was unable to suppress the phenotype. However, *HAL5* alone (plasmid pM89) suppressed the phenotype as well as did the original plasmid. A homolog of *HAL5*, called *HAL4* (plasmid pM73), also suppressed, although *HAL4* was not found in the screen. *HAL4* and *HAL5* encode Ser/Thr protein kinases that function upstream of the K^+ influx transporters, Trk1p and Trk2p (Mulet et al., 1999). High-copy-number *TRK1* in the *arl1* strain suppressed hygromycin-B sensitivity relatively weakly (data not shown), suggesting that mere overexpression of Trk1p is insufficient and that Trk1p needs to be regulated by the Hal proteins.

Plasmid pDH32 contained YFR038w, YFR039c, *SAP155* and *ERJ5*. *SAP155* alone (CB2643) suppressed as well as the original. Three other *SAP* genes, *SAP4*, *SAP185* and *SAP190*, have been isolated based on sequence homology. All four Sap proteins interact with Sit4p (Luke et al., 1996), a phosphatase required for progression through the cell cycle, specifically the G_1-S transition (Sutton et al., 1991). However, only *SAP155* suppressed the hygromycin-B-sensitive phenotype of the *arl1* mutant.

We also isolated several different library plasmids that suppressed weakly compared to *SAP155* and the *HAL* genes (Table 3). Each insert had at least two open reading frames, so the suppressor in each case was identified by subcloning. First, *ATC1* (also known as *LIC4*), a gene involved in regulation of responses of cells to Li^+ (Hemenway and Heitman, 1999) was isolated. Other work from our laboratory has shown that *arl1 atc1* double mutants are extremely sensitive to Li^+ (Munson et al., 2004). Second, *MIG1* (encoding a repressor of *ENA1* transcription) was isolated (Alepuz et al., 1997). *ENA1* encodes the major Na^+ efflux pump in cells (Garciadeblas et al., 1993; Marquez and Serrano, 1996). In addition, isolates were found that contained *CKI1* [encoding choline kinase (Kim and Carmen, 1999)], YJL193w (Paulsen et al., 1998) (encoding a protein with homology to Sly41p, a triose phosphate transporter) and *FUN21* (a gene with no known function).

HAL4, *HAL5* and *SAP155* are downstream effectors of *ARL1*

We next investigated homozygous diploid deletion strains missing genes identified as high-copy-number suppressors of the loss of *ARL1*. Because the *arl1* strain was hygromycin-B sensitive and this sensitivity was reversed by K^+ (Fig. 1), we

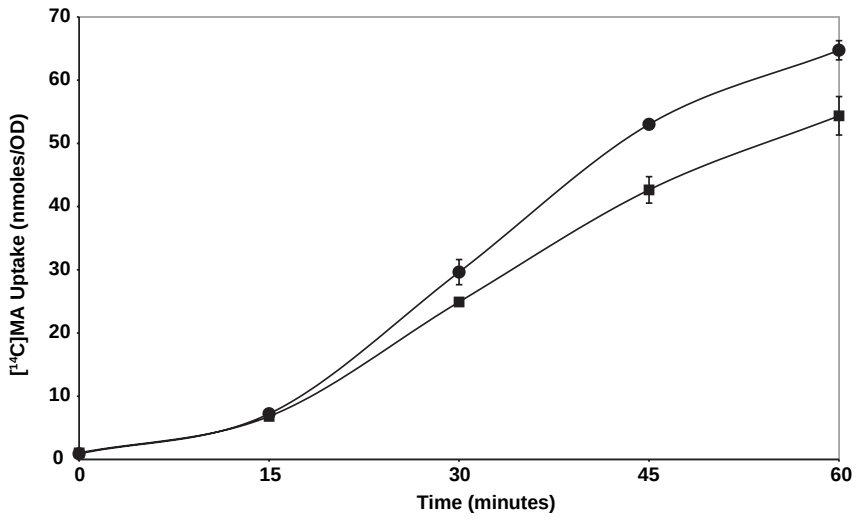

Fig. 3. Strains lacking *ARL1* accumulate [^{14}C]-MA. PSY316 (*ARL1*; squares) and MA03 (*arl1*; circles) were incubated with [^{14}C]-MA (2.5 μCi ml^{-1}, 2 mM final concentration) for up to 60 minutes. Cells were collected on nitrocellulose filters and washed three times with ice-cold 20 mM $MgCl_2$. Filters were counted by scintillation spectroscopy to determine the amount of internalized radioactivity. Data were collected in triplicate and are shown ±s.d. This experiment was repeated four times with similar results.

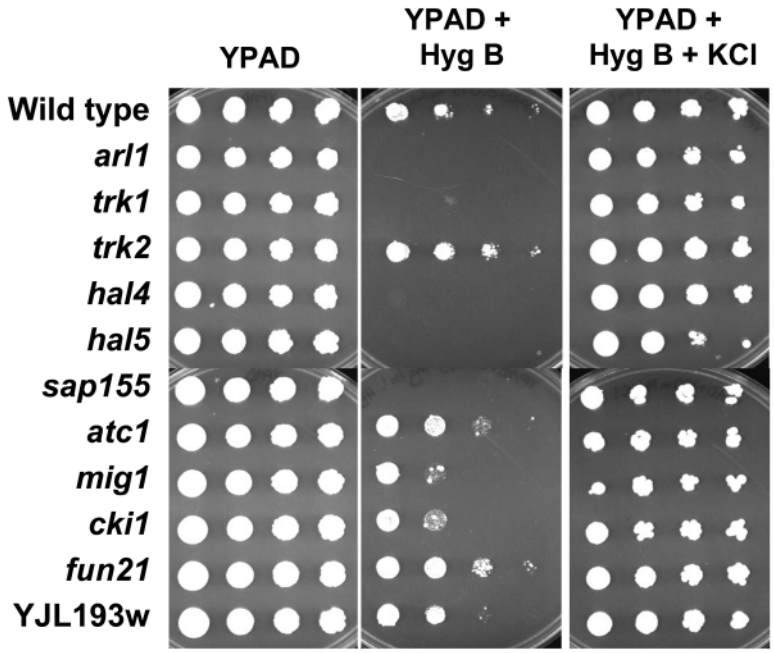

Fig. 4. Mutants deleted for genes obtained in the high-copy-number suppressor screen are variously sensitive to hygromycin B. Selected homozygous diploid deletion strains (from the Deletion Collection) were grown overnight in YPAD liquid medium to saturation. Serial tenfold dilutions in water were performed in a multiwell plate and then spotted onto solid medium with a replicator tool. Strains were tested on YPAD medium with or without 0.1 mg ml^{-1} hygromycin B and with or without 100 mM KCl. Cells grown in YPAD + KCl grew as well as cells on YPAD alone and are therefore not shown. Cells were grown for 4 days at 30°C then photographed.

screened the selected deletion strains on medium containing hygromycin B with and without KCl. The three deletion strains corresponding to the strong high-copy-number suppressors (the *hal4*, *hal5* and *sap155* mutants) were sensitive to hygromycin B (Fig. 4) and TMA (data not shown), and sensitivity was suppressed by K^+ (Fig. 4). It was previously shown that *hal4 hal5* and *trk1 trk2* double mutants are sensitive to hygromycin B (Madrid et al., 1998; Mulet et al., 1999). We tested both the *trk1* and the *trk2* homozygous diploid single mutant strains; only the *trk1* mutant was sensitive to hygromycin B (Fig. 4). Trk1p activity is expressed at higher levels than Trk2p activity under normal growth conditions (Ramos et al., 1994), suggesting that expression of *TRK1* in the *trk2* mutant was sufficient (Bertl et al., 2003).

The remaining five strains were not particularly sensitive to hygromycin B (Fig. 4) or TMA (data not shown) compared with the wild-type parent. Thus, the weak suppressors *ATC1*, *CKI1*, *FUN21*, *MIG1* and YJL193w did not appear to function downstream of *ARL1* and were assumed to be bypass suppressors. These were not studied further.

We next determined epistatic relationships among these genes. The *arl1*, *hal4*, *hal5* and *sap155* strains were transformed with *ARL1* (pARY1-3), *HAL5* (pM89) or *SAP155* (CB2643). Although the loss of *ARL1* was suppressed by *HAL4*, *HAL5* and *SAP155*, overexpression of *ARL1* was unable to suppress the loss of any of these genes, demonstrating that *HAL4*, *HAL5* and *SAP155* were downstream of *ARL1*. Furthermore, *HAL5* appeared to be downstream of *SAP155*, because *HAL5* suppressed the hygromycin-B-sensitive phenotype of the *sap155* mutant. Finally, *SAP155* failed to suppress the hygromycin-B-sensitive phenotype of the *hal5* mutant (data not shown), lending additional support to the notion that *HAL5* is downstream of *SAP155*.

arl1 mutants exhibit a rubidium uptake defect

The evidence cited above is consistent with a model in which *ARL1* regulates Trk1 K^+ transporter activity. To test this hypothesis, wild-type and *arl1* strains were incubated with $^{86}RbCl$. Rb^+, a congener of K^+, has been used extensively to examine K^+ homeostasis (Haro et al., 1993; Ko et al., 1990; Madrid et al., 1998; Mulet et al., 1999; Ramos et al., 1994; Yenush et al., 2002). Loss of *ARL1* resulted in decreased $^{86}Rb^+$ uptake (Fig. 5A). However, the decrease in $^{86}Rb^+$ influx by the *arl1* mutant was not as severe as that exhibited by a strain lacking *TRK1* but was more similar to the level of uptake observed in strains with deletions of the regulatory genes *HAL4* and especially *HAL5* (Fig. 5B). Loss of *ARL1* did not have an effect on $^{86}Rb^+$ efflux (data not shown).

arl1 mutants do not exhibit a proton efflux defect

Plasma membrane potential is controlled by regulation of both cation influx, driven in large measure by the K^+ transporters Trk1p and Trk2p, and by cation efflux, driven primarily by the H^+-efflux pump Pma1p (Gaber, 1992). Hyperpolarization can be caused by either a decrease in cation uptake or an increase in cation efflux. Although the results of the Rb^+ uptake experiment are consistent with a decrease in K^+ uptake being responsible for the membrane polarity defects of the *arl1* strain, this result does not exclude a role for altered cation efflux in this strain. To test the hypothesis that excess proton efflux contributes to the hyperpolarity observed in *arl1* cells in addition to the K^+ (Rb^+) influx defect (Fig. 5), a whole cell proton efflux assay was used. When resuspended in water and assayed using this protocol (Fig. 6), the *arl1* mutant (closed circles) was unable to acidify the external medium as well as the wild-type strain (closed squares), counter to the result predicted if proton efflux is contributing to hyperpolarity. However, under these conditions, both efflux and influx of H^+ occur (Perlin et al., 1988). To examine the effect of the *arl1* mutation on efflux only, the experiment was repeated in the presence of K^+ (Perlin et al., 1988). When cells were suspended in 25 mM

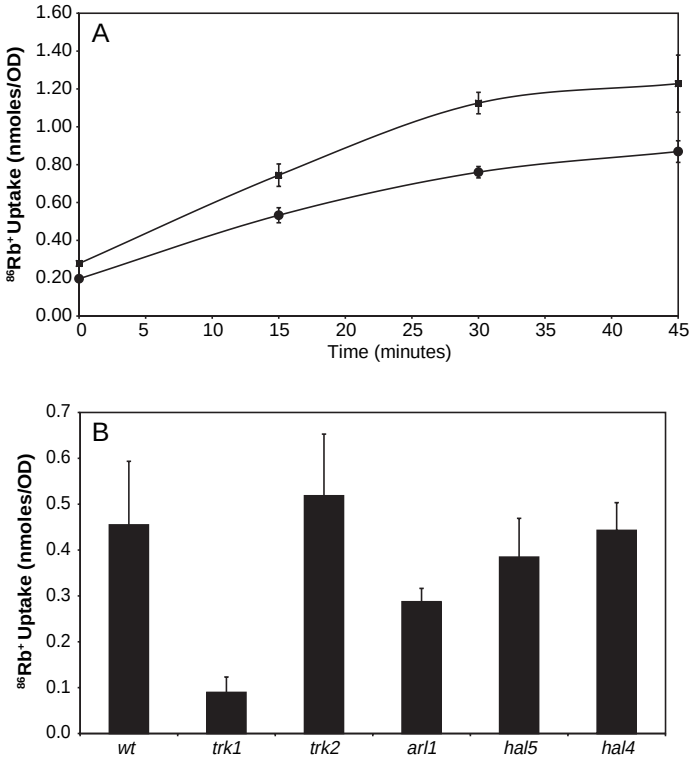

Fig. 5. Strains lacking *ARL1* have a $^{86}Rb^+$ uptake defect. (A) PSY316 (*ARL1*; squares) and MA03 (*arl1*; circles) were treated then incubated with ^{86}RbCl for up to 45 minutes as described in Materials and Methods. Uptake by each strain was measured in triplicate and data shown is the average±s.d. This experiment was repeated three times with similar results. (B) Selected strains from the Deletion Collection were treated then incubated with ^{86}RbCl for 0 minutes and 30 minutes as described in Materials and Methods. Uptake by each strain was measured in triplicate and data shown in the average difference ± s.d. between the 0 and 30 minute time points. This experiment was repeated twice with similar results.

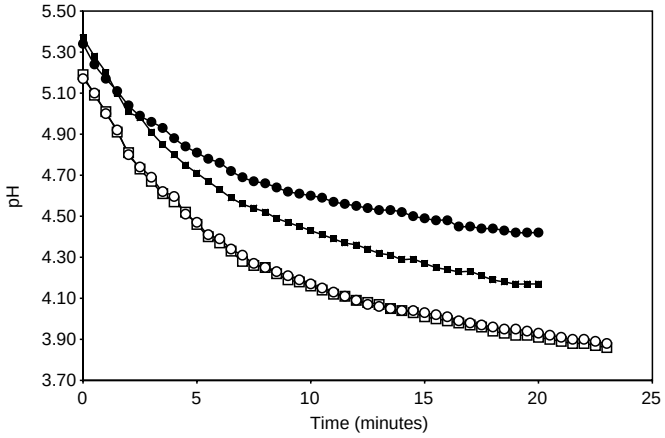

Fig. 6. Strains lacking *ARL1* do not have an apparent H^+ efflux defect. PSY316 (*ARL1*; squares) and MA03 (*arl1*; circles) were treated as described in Materials and Methods. Measurements made in the presence of 25 mM KCl are open symbols and without KCl are closed symbols. Proton efflux was determined by measuring the change in pH after initiating the reaction with glucose. This experiment was repeated three times with similar results.

KCl, both strains exhibited an apparent increase in proton efflux and, importantly, the difference in proton efflux between the two strains was eliminated (Fig. 6, open symbols). The difference in steady-state pH level in the absence of added K^+ between the *arl1* and wild-type strains can therefore be attributed to increased H^+ uptake by the *arl1* mutant and not decreased H^+ efflux. This result is completely consistent with our earlier observation that the *arl1* strain is sensitive to low pH. In summary, these results suggest that *ARL1* regulates membrane potential via regulation of K^+ influx, rather than by regulation of H^+ efflux.

Loss of *Arl1* affects neither the steady-state level of Trk1p nor its localization

The results shown above suggest two different models. First, loss of *ARL1* could result in decreased levels of Trk proteins at the plasma membrane. This hypothesis is consistent with the known role of Arl1p in regulation of membrane traffic (Bonangelino et al., 2002; Jochum et al., 2002; Panic et al., 2003; Rosenwald et al., 2002; Setty et al., 2003). Second, loss of *ARL1* could result in misregulation of a signaling cascade that regulates the activity of the Trk proteins. This hypothesis is consistent with our findings that *HAL4* and *HAL5* in high copy number suppress the hygromycin-B-sensitive phenotype of the *arl1* strain.

To begin to discover which of these models was correct, we verified that *ARL1* functions upstream of *TRK1*. To do this, an *arl1 trk1* double mutant was constructed. $^{86}Rb^+$ analysis (as in Fig. 5) demonstrated that this mutant had similar levels of uptake to the isogenic *ARL1 trk1* mutant, suggesting that *ARL1* and *TRK1* function in the same genetic pathway (data not shown).

Subsequently, we constructed strains bearing a Myc-epitope-tagged *TRK1* allele at the *TRK1* chromosomal locus in both wild-type and *arl1* backgrounds. The tagged allele, containing 13 Myc epitopes fused to the C-terminus of Trk1p, was functional by all measurements. First, when comparing cells with wild-type *TRK1* and *TRK1-Myc*, the strains grow at the same rate as wild-type in the absence of added K^+ (*trk1* mutants are K^+ bradytrophs). Second, $^{86}Rb^+$ uptake (as in Fig. 5) was unaffected. Finally, the *TRK1-Myc ARL1* strain was no more sensitive to hygromycin B than the *TRK1 ARL1* strain. Similarly, the *TRK1-Myc arl1* strain was indistinguishable from the *TRK1 arl1* strain (data not shown).

As shown in Fig. 7, western blot analysis with an anti-Myc antibody revealed a 180 kDa band. This band is only present in cells bearing the *TRK1-Myc* allele, not cells containing wild-type *TRK1* (data not shown). Loss of *ARL1* did not appear to affect the steady-state levels of Trk1-Myc in cells. Subcellular fractionation using differential centrifugation was then performed. Similar amounts of Trk1-Myc were present in the P14 fractions of both *ARL1* and *arl1* strains, where both the plasma membrane marker Pma1p and the vacuolar membrane marker Vph1p are found. These results suggest that Arl1p might be important for regulating the activity of Trk1p at the plasma membrane, rather than regulating delivery of Trk1p to the plasma membrane.

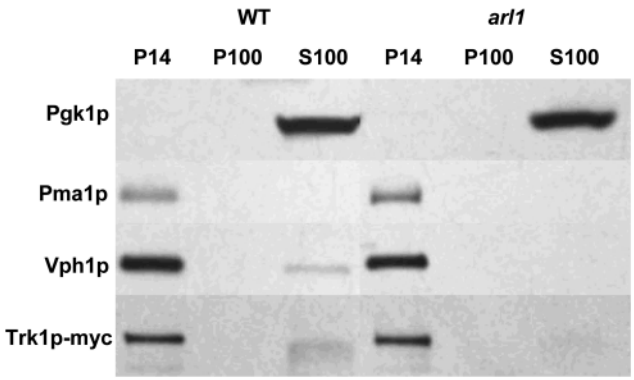

Fig. 7. Strains lacking *ARL1* do not mislocalize Trk1-Myc. AM300 (*TRK1-Myc ARL1*) and AM310 (*TRK1-Myc arl1*) were grown in SD medium with supplements to mid-log phase and then harvested by centrifugation and prepared for subcellular fractionation as described in Materials and Methods. Equal numbers of cell equivalents (as measured by OD_{600}), after spheroplasting and lysis, were fractionated by differential centrifugation. After addition of SDS-PAGE sample buffer followed by heating to 42°C for 5 minutes, samples were loaded onto a 10% polyacrylamide gel. After electrophoresis, the proteins were electroblotted to nitrocellulose, then used for western-blot analysis with anti-Pgk1p antibody (top panel; a cytosol marker), anti-Pma1p antibody (second panel; a plasma membrane marker), anti-Vph1p antibody (third panel; vacuole marker) or anti-Myc antibody to detect Trk1p-Myc (bottom panel).

Discussion

Investigations into the functions of the Arl family began in 1991 with the identification of the *arf-like* gene of *D. melanogaster* (Tamkun et al., 1991). Many Arl genes have been identified in many different organisms since, demonstrating, first, that this gene family is highly conserved across eukaryotic evolution and, second, that family members are more divergent than are members of the related Arf family. Although many *ARL* genes have been identified, the functions of Arl proteins remain relatively unexplored. Data from our laboratory and others have shown that Arl1 is a regulator of membrane traffic in yeast (Bonangelino et al., 2002; Jochum et al., 2002; Rosenwald et al., 2002) and mammalian cells (Eboue et al., 1998; Icard-Liepkalns et al., 1997; Lowe et al., 1996; Lu et al., 2001; Van Valkenburgh et al., 2001). However, in this study, yeast Arl1 was found to have a novel role in regulation of K^+ influx that does not appear to be a result of Arl1p's ability to control membrane traffic.

Mutations in several genes result in sensitivity to toxic cations, including hygromycin B. The genes include those coding for calcineurin [the A (catalytic) subunits encoded by *CNA1* and *CNA2*, and the B (regulatory) subunit encoded by *CNB1*] (Withee et al., 1998), the plasma membrane K^+ transporters Trk1p and Trk2p (Mulet et al., 1999), the protein kinases Hal4p and Hal5p (which regulate Trk1p and Trk2p) (Mulet et al., 1999), Nhx1p (the Na^+/H^+ exchanger at the pre-vacuolar membrane) (Gaxiola et al., 1999), Gef1p (the chloride channel at the pre-vacuolar membrane) (Gaxiola et al., 1999), and Pmp3 (a small hydrophobic peptide) (Navarre and Goffeau, 2000). Mutations in these genes are thought to result in hyperpolarization of the plasma membrane, leading to increased uptake of toxic cations (Goossens et al., 2000), as we observed in the *arl1* mutant. In several of these strains, specifically the *hal4 hal5* and *trk1 trk2* double mutants, sensitivity to toxic cations like hygromycin B can be suppressed by the addition of K^+ (Haro et al., 1993; Madrid et al., 1998; Mulet et al., 1999), an effect also observed in the *arl1* mutant. By contrast, mutations in other genes, including *PMA1* (Goossens et al., 2000; Perlin et al., 1988; Perlin et al., 1989; Serrano et al., 1986) and the phosphatases *PPZ1* and *PPZ2* (Yenush et al., 2002), led to toxic cation tolerance and decreased potential across the plasma membrane. Because the hyperpolarization phenotype of the *arl1* mutant is reversed by the inclusion of a depolarizing cation, K^+, our findings are consistent with a model in which the plasma membrane of the *arl1* strain is hyperpolarized as a result of disturbances in K^+ influx rather than H^+ efflux.

We identified three suppressors that are likely to function in the same pathway as *ARL1 – HAL4*, *HAL5* and *SAP155*. The molecular function of *SAP155* is not well understood at present. All four of the Sap proteins (Sap4p, Sap155p, Sap185p and Sap190p) interact with the phosphatase, Sit4p, a regulator of the G_1-S transition. Sit4p exists as a monomer in early G_1 phase but becomes associated with Sap155p and, in a separate complex, with Sap190p by the end of G_1, and the complexes remain together until mid-M-phase (Sutton et al., 1991). The binding of Sap190 and Sap155 to Sit4 appear to be mutually exclusive, and overexpression of *SAP155* cannot suppress the double deletion of both *SAP190* and its relative *SAP185*, suggesting that the functions Sap155p provides are different from the functions of Sap185p and Sap190p (Luke et al., 1996). The Sap proteins might be substrates for Sit4p, because they are hyperphosphorylated in the absence of Sit4p (Luke et al., 1996). It has also been observed that overexpression of only *SAP155*, but not any of the other three *SAP* genes, protects cells against *Kluyveromyces lactis* zymocin, an inhibitor that prevents progression through G_1 to S (Jablonowski et al., 2001). Furthermore, functional Sit4p is required for expression of the zymocin-sensitivity phenotype (Jablonowski et al., 2001).

Our results with *SAP155* suggest a link between regulation of ion homeostasis and regulation of the cell cycle, which has been explored by others. *SIT4* expression is induced by Li^+, Na^+ and K^+, and overexpression of *SIT4* confers resistance to Li^+ (Masuda et al., 2000). *SIT4* overexpression stimulates Rb^+ efflux and causes a rise in intracellular pH. The Ppz phosphatases also help to regulate the responses of cells to Na^+, H^+ and K^+ (Yenush et al., 2002). Deletion of both *PPZ* genes renders cells tolerant to a range of toxic cations including Li^+, Na^+, spermine, TMA and hygromycin B. Cation tolerance depends on the presence of functional *TRK1* and *TRK2*. Overexpression of *PPZ1*, by contrast, renders cells defective for Rb^+ uptake and confers a slow-growth phenotype, implying that it is a negative regulator of cell cycle progression. The slow growth phenotype, however, is partially suppressed by inclusion of extra K^+ in medium. Finally, Ppz1p and Sit4p appear to have opposing functions in regulation of the cell cycle (Clotet et al., 1999). Thus, regulation of ion homeostasis is important for cell cycle regulation and might explain the connection we observed between *SAP155* and *ARL1*, an observation we continue to explore. Specifically, we are testing the hypothesis that overexpression of *SAP155* leads to decreased K^+ efflux in a manner that depends on *SIT4* and

TOK1, the K^+ efflux channel (Bertl et al., 2003; Zhou et al., 1995).

Uptake of K^+ by yeast is accomplished by the transporters Trk1p and Trk2p under normal growth conditions (Gaber et al., 1988). Trk2p shares 55% amino acid identity with Trk1 (Ko and Gaber, 1991). It was initially suggested that each has 12 membrane spans (Gaber et al., 1988; Ko and Gaber, 1991) but, more recently, it has been suggested that each Trk protein instead contains four repeats of a membrane-span/P-loop/membrane-span motif, each repeat similar in structure to the K^+ channel KcsA, from *Streptomyces lividans* (Haro and Rodriguez-Navarro, 2002). Loss of both *TRK* genes results in a conditional lethal phenotype suppressed by K^+ (Ko and Gaber, 1991; Madrid et al., 1998). Nonspecific uptake of K^+ through other transporters permits growth under these conditions. One nonspecific channel is NSC1, which has been described electrochemically, although the gene(s) that encodes this activity has not yet been identified (Bihler et al., 1998; Bihler et al., 2002). Interestingly, hygromycin B blocks this channel (Bihler et al., 2002).

TRK1 encodes a high-affinity K^+ transporter (Gaber et al., 1988), whereas *TRK2* (dispensable in the presence of *TRK1*) encodes a transporter of moderate affinity (Ramos et al., 1994). Trk2p in addition appears to mediate an inward proton current that is regulated by extracellular pH (Bihler et al., 1999). The *trk1 trk2* strains are sensitive to hygromycin B and other cations, and exhibit hyperpolarization of the plasma membrane (Madrid et al., 1998). The *hal4 hal5* mutants exhibit similar but milder phenotypes (Mulet et al., 1999). As we demonstrate here, the *arl1* mutant was similar to the *hal4 hal5* double mutant (Mulet et al., 1999) and to the *hal4* and *hal5* single mutants.

HAL4 (also called *SAT4*) and *HAL5* encode Ser/Thr kinases that are partially redundant, although we observed that the single mutant strains were sensitive to hygromycin B and TMA. In addition, the single mutants take up less $^{86}Rb^+$ than the wild type. The function of the two Hal proteins in regulating ion stress depends on the presence of *TRK1* and *TRK2* (Mulet et al., 1999), demonstrating that they act upstream of *TRK1* and *TRK2*. We have shown here that *ARL1* is upstream of the *HAL* genes and upstream of *TRK1*. *HAL* gene overexpression leads to toxic cation tolerance, suggesting that these proteins alter the affinity of Trk1p and Trk2p for K^+ and for toxic ions such as Na^+ and Li^+. *HAL4* and *HAL5* are members of the Npr1 subfamily of kinases (Hunter and Plowman, 1997). This subgroup includes other regulators of membrane transport, including Npr1p, which regulates the Gap1p amino acid permease (Stanbrough and Magasanik, 1995), and Ptk2p and YOR267c, which regulate Pma1p, the H^+-ATPase (Goossens et al., 2000). Our genetic evidence linking *ARL1* to *HAL4* and *HAL5* thus suggests that *ARL1* acts as an upstream regulator of K^+ influx via regulation of *HAL4* and *HAL5*.

These data are consistent with a model in which Arl1p is a positive regulator of the positive regulators Hal4p and Hal5p. Because we found that the inclusion of high-copy-number *TRK1* suppressed the hygromycin-B sensitivity of the *arl1* mutant weakly, the presence of Trk1 is not sufficient; rather, the transporter must be in the high-affinity form. Our data are not consistent with a model in which Arl1p controls delivery of Trk1p to the plasma membrane, because loss of *ARL1* has no effect on the steady-state level of Trk1-Myc, nor on the fraction in which it resides. Although the P14 fraction contains both vacuolar and plasma membranes, and this analysis does not distinguish between the two, we can assert that there is not substantial Trk1-Myc in internal membranes (Golgi, secretory vesicles and endosomes) in the *arl1* mutant strain because virtually no Trk1-Myc was observed in the P100 fraction.

In summary, we demonstrate here that Arl1p, a member of the Arl family of proteins, has a novel role in ion homeostasis and propose that it contributes to K^+ influx via regulation of Trk proteins. Despite Arl1p's documented role in regulation of membrane traffic, our results suggest that Arl1p's role in regulation of K^+ influx proceeds by a mechanism other than by regulation of Trk delivery to the plasma membrane. Future experiments will test the hypothesis that *ARL1* encodes a regulator of a phosphorylation cascade that involves the Hal kinases and the Trk K^+ transporters.

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References

Adams, A., Gottschling, D. E., Kaiser, C. A. and Stearns, T. (1997). *Methods in Yeast Genetics: A Cold Spring Harbor Laboratory Course Manual*. Plainview, NY: Cold Spring Harbor Laboratory Press.

Alepuz, P. M., Cunningham, K. W. and Estruch, F. (1997). Glucose repression affects ion homeostasis in yeast through the regulation of the stress-activated *ENA1* gene. *Mol. Microbiol.* **26**, 91-98.

Anderson, M. S., Yarger, J. G., Burck, C. L. and Poulter, C. D. (1989). Farnesyl diphosphate synthetase. Molecular cloning, sequence, and expression of an essential gene from *Saccharomyces cerevisiae*. *J. Biol. Chem*. **264**, 19176-19184.

Baudin, A., Ozier-Kalogeropoulos, O., Denouel, A., Lacroute, F. and Cullin, C. (1993). A simple and efficient method for direct gene deletion in *Saccharomyces cerevisiae*. *Nucleic Acids Res.* **21**, 3329-3330.

Bertl, A., Ramos, J., Ludwig, J., Lichtenberg-Frate, H., Reid, J., Bihler, H., Calero, F., Martinez, P. and Ljungdahl, P. O. (2003). Characterization of potassium transport in wild-type and isogenic yeast strains carrying all combinations of *trk1*, *trk2*, and *tok1* null mutations. *Mol. Microbiol.* **47**, 767-780.

Bihler, H., Gaber, R. F., Slayman, C. L. and Bertl, A. (1999). The presumed potassium carrier Trk2p in *Saccharomyces cerevisiae* determines an H^+-dependent, K^+-independent current. *FEBS Lett.* **447**, 115-120.

Bihler, H., Slayman, C. L. and Bertl, A. (2002). Low-affinity potassium uptake by *Saccharomyces cerevisiae* is mediated by NSC1, a calcium-blocked non-specific cation channel. *Biochim. Biophys. Acta* **1558**, 109-118.

Bihler, H., Slayman, C. L. and Bertl, A. (1998). NSC1: a novel high-current inward rectifier for cations in the plasma membrane of *Saccharomyces cerevisiae*. *FEBS Lett*. **432**, 59-64.

Bonangelino, C. J., Chavez, E. M. and Bonifacino, J. S. (2002). Genomic screen for vacuolar protein sorting genes in *Saccharomyces cerevisiae*. *Mol. Biol. Cell* **13**, 2486-2501.

Brodersen, D. E., Clemons, W. M., Jr, Carter, A. P., Morgan-Warren, R. J., Wimberly, B. T. and Ramakrishnan, V. (2000). The structural basis for the action of the antibiotics tetracycline, pactamycin, and hygromycin B on the 30S ribosomal subunit. *Cell* **103**, 1143-1154.

Clotet, J., Gar, E., Aldea, M. and Arino, J. (1999). The yeast Ser/Thr phosphatases Sit4 and Ppz1 play opposite roles in regulation of the cell cycle. *Mol. Cell. Biol.* **19**, 2408-2415.

Eboue, D., Icard-Liepkalns, C., Beringer, T. M. and Liepkalns, V. A. (1998). Attenuation of 19-9 antigen secretion in human colorectal carcinoma cell cultures by transfection with cDNA encoding novel ADP-ribosylation factor-like proteins. *Arch. Biochem. Biophys.* **350**, 145-156.

Gaber, R. F. (1992). Molecular genetics of yeast ion transport. *Int. Rev. Cytol.* **137A**, 299-353.

Gaber, R. F., Styles, C. A. and Fink, G. R. (1988). *TRK1* encodes a plasma membrane protein required for high-affinity potassium transport in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **8**, 2848-2859.

Garciadeblas, B., Rubio, F., Quintero, F. J., Baneulos, M. A., Haro, R. and Rodriguez-Navarro, A. (1993). Differential expression of two genes encoding isoforms of the ATPase involved in sodium efflux in *Saccharomyces cerevisiae*. *Mol. Gen. Genet.* **236**, 363-368.

Gaxiola, R. A., Rao, R., Sherman, A., Grisafi, P., Alper, S. L. and Fink, G. R. (1999). The *Arabidopsis thaliana* proton transporters, AtNhx1 and Avp1, can function in cation detoxification in yeast. *Proc. Natl. Acad. Sci. USA* **96**, 1480-1485.

Gaynor, E. C. and Emr, S. D. (1997). COPI-independent anterograde transport: cargo-selective ER to Golgi protein transport in yeast *COPI* mutants. *J. Cell Biol.* **136**, 789-802.

Goossens, A., de la Fuente, N., Forment, J., Serrano, R. and Portillo, F. (2000). Regulation of yeast H^+-ATPase by protein kinases belonging to a family dedicated to activation of plasma membrane transporters. *Mol. Cell. Biol.* **20**, 7654-7661.

Haro, R., Banuelos, M. A., Quintero, F. J., Rubio, F. and Rodriguez-Navarro, A. (1993). Genetic basis of sodium exclusion and sodium tolerance in yeast. A model for plants. *Physiol. Plant.* **89**, 868-874.

Haro, R. and Rodriquez-Navarro, A. (2002). Molecular analysis of the mechanism of potassium uptake through the TRK1 transporter of *Saccharomyces cerevisiae*. *Biochim. Biophys. Acta* **1564**, 114-122.

Hemenway, C. S. and Heitman, J. (1999). Lic4, a nuclear phosphoprotein that cooperates with calcineurin to regulate cation homeostasis in *Saccharomyces cerevisiae*. *Mol. Gen. Genet.* **261**, 388-401.

Hill, J. E., Myers, A. M., Koerner, T. J. and Tzagoloff, A. (1986). Yeast/*E. coli* shuttle vectors with multiple unique restriction sites. *Yeast* **2**, 163-167.

Hoffman, C. S. and Winston, F. (1987). A ten-minute DNA preparation from yeast efficiently releases autonomous plasmids for transformation of *Escherichia coli*. *Gene* **57**, 267-272.

Hunter, T. and Plowman, G. D. (1997). The protein kinases of budding yeast: six score and more. *Trends Biochem. Sci.* **22**, 18-22.

Hwang, Y.-W. and Miller, D. L. (1987). A mutation that alters the nucleotide specificity of elongation factor Tu, a GTP regulatory proteins. *J. Biol. Chem.* **262**, 13081-13085.

Icard-Liepkalns, C., Ravassard, P., Liepkalns, V. A., Chatail, F. and Mallet, J. (1997). An ADP-ribosylation factor (ARF)-like protein involved in regulated secretion. *Eur. J. Biochem.* **246**, 388-393.

Ito, H., Fukuda, Y., Murata, K. and Kimura, A. (1983). Transformation of intact yeast cells treated with alkali cations. *J. Bacteriol.* **153**, 163-168.

Jablonowski, D., Butler, A. R., Fichtner, L., Gardiner, D., Schaffrath, R. and Stark, M. J. R. (2001). Sit4p protein phosphatase is required for sensitivity of *Saccharomyces cerevisiae* to *Kluyveromyces lactis* zymocin. *Genetics* **159**, 1479-1489.

Jochum, A., Jackson, D., Schwarz, H., Pipkorn, R. and Singer-Kruger, B. (2002). Yeast Ysl2p, homologous to Sec7 domain guanine nucleotide exchange factors, functions in endocytosis and maintenance of vacuole integrity and interacts with the Arf-like small GTPase Arl1p. *Mol. Cell. Biol.* **22**, 4914-4928.

Jones, S., Litt, R. J., Richardson, C. J. and Segev, N. (1995). Requirement of nucleotide exchange factor for Ypt1 GTPase mediated protein transport. *J. Cell Biol.* **130**, 1051-1061.

Kim, K. H. and Carman, G. M. (1999). Phosphorylation and regulation of choline kinase from *Saccharomyces cerevisiae* by protein kinase A. *J. Biol. Chem.* **274**, 9531-9518.

Ko, C. H., Buckley, A. M. and Gaver, R. F. (1990). TRK2 is required for low affinity K^+ transport in *Saccharomyces cerevisiae. Genetics* **125**, 305-312.

Ko, C. H. and Gaber, R. F. (1991). *TRK1* and *TRK2* encode structurally related K^+ transporters in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **11**, 4266-4273.

Lee, F.-J. S., Huang, C.-F., Yu, W.-L., Buu, L.-M., Lin, C.-Y., Huang, M.-C., Moss, J. and Vaughan, M. (1997). Characterization of an ADP-ribosylation factor-like 1 protein in *Saccharomyces cerevisiae*. *J. Biol. Chem.* **272**, 30998-31005.

Longtine, M. S., McKenzie, A., Demarini, D. J., Shah, N. G., Wach, A., Brachat, A., Philippsen, P. and Pringle, J. R. (1998). Additional modules for versatile and economical PCR based gene deletion and modification in *Saccharomyces cerevisiae*. *Yeast* **14**, 953-961.

Lowe, S. L., Wong, S. H. and Hong, W. (1996). The mammalian ARF-like protein 1 (Arl1) is associated with the Golgi complex. *J. Cell Sci.* **109**, 209-220.

Lu, L. and Hong, W. (2003). Interaction of Arl1-GTP with GRIP domains recruits autoantigens golgin-97 and golgin-245/p230 onto the Golgi. *Mol. Biol. Cell* **14**, 3767-3781.

Lu, L., Horstmann, H., Ng, C. and Hong, W. (2001). Regulation of Golgi structure and function by ARF-like protein 1 (Arl1). *J. Cell Sci.* **114**, 4534-4555.

Luke, M. M., Della Seta, F., di Como, C. J., Sugimoto, H., Kobayashi, R. and Arndt, K. T. (1996). The SAPs, a new family of proteins, associate and function positively with the SIT4 phosphatase. *Mol. Cell. Biol.* **16**, 2744-2755.

Madrid, R., Gomez, M. J., Ramos, J. and Rodriguez-Navarro, A. (1998). Ectopic potassium uptake in *trk1 trk2* mutants of *Saccharomyces cerevisiae* correlates with a highly hyperpolarized membrane potential. *J. Biol. Chem.* **273**, 14838-14844.

Marquez, J. A. and Serrano, R. (1996). Multiple transduction pathways regulate the sodium-extrusion gene *PMR2/ENA1* during salt stress in yeast. *FEBS Lett.* **382**, 89-92.

Masuda, C. A., Ramirez, J., Pena, A. and Montero-Lomeli, M. (2000). Regulation of monovalent ion homeostasis and pH by the Ser-Thr protein phosphatase SIT4 in *Saccharomyces cerevisiae*. *J. Biol. Chem.* **275**, 30957-30961.

Mendoza, I., Quintero, F. J., Bressan, R. A., Hasegawa, P. M. and Pardo, J. M. (1996). Activated calcineurin confers high tolerance to ion stress and alters the budding pattern and cell morphology of yeast cells. *J. Biol. Chem.* **271**, 23061-23067.

Mulet, J. M., Leube, M. P., Kron, S. J., Rios, G., Fink, G. R. and Serrano, R. (1999). A novel mechanism of ion homeostasis and salt tolerance in yeast: the Hal4 and Hal5 protein kinases modulate the Trk1-Trk2 potassium transporter. *Mol. Cell. Biol.* **19**, 3328-3337.

Munson, A. M., Love, S. L., Shu, J., Palanivel, V. R. and Rosenwald, A. G. (2004). *ARL1* participates with *ATC1/LIC4* to regulate responses of yeast cells to ions. *Biochem. Biophys. Res. Comm.* **315**, 617-623.

Nasmyth, K. A. and Reed, S. I. (1980). Isolation of genes by complementation in yeast: molecular cloning of a cell-cycle gene. *Proc. Natl. Acad. Sci. USA* **77**, 2119-2123.

Navarre, C. and Goffeau, A. (2000). Membrane hyperpolarization and salt sensitivity induced by deletion of PMP3, a highly conserved small protein of yeast plasma membrane. *EMBO J.* **19**, 2515-2524.

Panic, B., Whyte, J. R. and Munro, S. (2003). The ARF-like GTPases Arl1p and Arl3p act in a pathway that interacts with vesicle-tethering factors at the Golgi apparatus. *Curr. Biol.* **13**, 405-410.

Paulsen, I. T., Sliwinski, M. K., Nelissen, B., Goffeau, A. and Saier, M. H., Jr (1998). Unified inventory of established and putative transporters encoded within the complete genome of *Saccharomyces cerevisiae*. *FEBS Lett.* **430**, 116-125.

Perlin, D. S., Brown, C. L. and Haber, J. E. (1988). Membrane potential defect in hygromycin B-resistant *pma1* mutants of *Saccharomyces cerevisiae*. *J. Biol. Chem.* **263**, 18118-18122.

Perlin, D. S., Harris, S. L., Seto-Young, D. and Haber, J. E. (1989). Defective H^+-ATPase of hygromycin B-resistant *pma1* mutants from *Saccharomyces cerevisiae*. *J. Biol. Chem.* **264**, 21857-21864.

Peterson, G. L. (1977). A simplification of the protein assay method of Lowry et al. which is more generally applicable. *Anal. Biochem.* **83**, 346-356.

Powers, S., Gonzales, E., Christensen, T., Cubert, J. and Broek, D. (1991). Functional cloning of BUD5, and CDC25-related gene from *S. cerevisiae* that can suppress a dominant-negative RAS2 mutant. *Cell* **65**, 1225-1231.

Powers, S., O'Neill, K. and Wigler, M. (1989). Dominant yeast and mammalian RAS mutants that interfere with the CDC25-dependent activation of wild-type RAS in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **9**, 390-395.

Ramos, J., Aligo, R., Haro, R. and Rodriguez-Navarro, A. (1994). TRK2 is not a low-affinity potassium transport in *Saccharomyces cerevisiae*. *J. Bacteriol*. **176**, 249-252.

Rosenwald, A. G., Rhodes, M. A., van Valkenburgh, H., Palanivel, V., Chapman, G., Boman, A., Zhang, C.-J. and Kahn, R. A. (2002). ARL1 and membrane traffic in *Saccharomyces cerevisiae*. *Yeast* **19**, 1039-1056.

Serrano, R. (1996). Salt tolerance in plants and microorganisms: toxicity targets and defense responses. *Int. Rev. Cytol*. **165**, 1-52.

Serrano, R., Kielland-Brandt, M. C. and Fink, G. R. (1986). Yeast plasma membrane ATPase is essential for growth and has homology with (Na^+ + K^+), K^+- and Ca^{2+}-ATPases. *Nature* **319**, 689-693.

Setty, S. R., Shin, M. E., Yoshino, A., Marks, M. S. and Burd, C. G. (2003). Golgi recruitment of GRIP domain proteins by Arf-like GTPase 1 Is regulated by Arf-like GTPase 3. *Curr. Biol*. **13**, 401-404.

Sherman, F., Fink, G. and Lawrence, C. (1974). *Methods in Yeast Genetics*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Soupene, E., Ramirez, R. M. and Kustu, S. (2001). Evidence that fungal MEP proteins mediate diffusion of the uncharged species NH_3 across the cytoplasmic membrane. *Mol. Cell. Biol.* **21**, 5733-5741.

Stanbrough, M. and Magasanik, B. (1995). Transcriptional and posttranscriptional regulation of the general amino acid permease of *Saccharomyces cerevisiae*. *J. Bacteriol*. **177**, 94-102.

Stearns, T., Willingham, M. C., Botstein, D. and Kahn, R. A. (1990). ADP-ribosylation factor is functionally and physically associated with the Golgi complex. *Proc. Natl. Acad. Sci. USA* **87**, 1238-1242.

Sutton, A., Immanuel, D. and Arndt, K. T. (1991). The SIT4 protein phosphatase functions in late G1 for progression into S phase. *Mol. Cell. Biol*. **11**, 2133-2148.

Szkopinska, A., Swiezewska, E. and Karst, F. (2000). The regulation of activity of main mevalonic acid pathway enzymes: farnesyl diphosphate synthase, 3-hydroxy-3-methylglutaryl-CoA reductase, and squalene synthase in yeast *Saccharomyces cerevisiae*. *Biochem. Biophys. Res. Comm.* **267**, 473-477.

Tamkun, J. W., Kahn, R. A., Kissinger, M., Brizuela, B. J., Rulka, C., Scott, M. P. and Kennison, J. A. (1991). The *arflike* gene encodes an essential GTP-binding protein in *Drosophila*. *Proc. Natl. Acad. Sci. USA* **88**, 3120-3124.

Tanida, I., Hasegawa, A., Iida, H., Ohya, Y. and Anraku, Y. (1995). Cooperation of calcineurin and vacuolar H^+-ATPase in intracellular Ca^{2+} homeostasis of yeast cells. *J. Biol. Chem*. **270**, 10113-10119.

Van Valkenburgh, H., Shern, J. F., Sharer, J. D., Zhu, X. and Kahn, R. A. (2001). ADP-ribosylation factors (ARFs) and ARF-like 1 (ARL1) have both specific and shared effectors: characterizing Arl1 binding proteins. *J. Biol. Chem*. **276**, 22826-22837.

Winzeler, E. A., Shoemaker, D. D., Astromoff, A., Liang, H., Anderson, K., Andre, B., Bangham, R., Benito, R., Boeke, J. D., Bussey, H. et al. (1999). Functional characterization of the *S. cerevisiae* genome by gene deletion and parallel analysis. *Science* **285**, 901-906.

Withee, J. L., Sen, R. and Cyert, M. S. (1998). Ion tolerance of *Saccharomyces cerevisiae* lacking the Ca^{2+}/CaM-dependent phosphatase (calcineurin) is improved by mutations in *URE2* or *PMA1*. *Genetics* **149**, 865-878.

Yenush, L., Mulet, J. M., Arino, J. and Serrano, R. (2002). The Ppz protein phosphatases are key regulators of K^+ and pH homeostasis: implications for salt tolerance, cell wall integrity and cell cycle progression. *EMBO J*. **21**, 920-929.

Zhou, X.-L., Vaillant, B., Loukin, S. H., Kung, C. and Saimi, Y. (1995). *YKC1* encodes the depolarization-activated K^+ channel in the plasma membrane of yeast. *FEBS Lett*. **373**, 170-176.