Biochimica et Biophysica Acta 1828 (2013) 623–631

Contents lists available at SciVerse ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamem

# Vhc1, a novel transporter belonging to the family of electroneutral cation–$Cl^-$ cotransporters, participates in the regulation of cation content and morphology of *Saccharomyces cerevisiae* vacuoles

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ARTICLE INFO

*Article history:*
Received 3 July 2012
Received in revised form 12 September 2012
Accepted 19 September 2012
Available online 27 September 2012

*Keywords:*
Salt tolerance
Yeast vacuole
Potassium homeostasis
Cation–chloride cotransport

ABSTRACT

Cation–$Cl^-$ cotransporters (CCCs) are integral membrane proteins which catalyze the coordinated symport of $Cl^-$ with $Na^+$ and/or $K^+$ ions in plant and mammalian cells. Here we describe the first *Saccharomyces cerevisiae* CCC protein, encoded by the *YBR235w* open reading frame. Subcellular localization studies showed that this yeast CCC is targeted to the vacuolar membrane. Deletion of the *YBR235w* gene in a salt-sensitive strain (lacking the plasma-membrane cation exporters) resulted in an increased sensitivity to high KCl, altered vacuolar morphology control and decreased survival upon hyperosmotic shock. In addition, deletion of the *YBR235w* gene in a mutant strain deficient in $K^+$ uptake produced a significant growth advantage over the parental strain under $K^+$-limiting conditions, and a hypersensitivity to the exogenous $K^+/H^+$ exchanger nigericin. These results strongly suggest that we have identified a novel yeast vacuolar ion transporter mediating a $K^+$–$Cl^-$ cotransport and playing a role in vacuolar osmoregulation. Considering its identified function, we propose to refer to the yeast *YBR235w* gene as *VHC1* (vacuolar protein homologous to CCC family 1).



## 1. Introduction

A relatively stable internal milieu is necessary for the optimal function of all types of cells. Among other things, cells need to regulate their intracellular concentrations of cations, anions and protons. For this purpose, they possess a broad variety of plasma-membrane and organellar transporters that mediate the fluxes of ions with differing mechanisms and capacities. The malfunction or absence of ion transporters (pumps, channels, antiporters and symporters) might be lethal for microorganisms that cannot cope with sudden environmental osmolarity changes. In mammalian cells, the malfunction of ion transporters results in severe diseases, and it has a detrimental effect on the quality of various agricultural crop species.

Many recent advances in identifying ion transport systems and their regulators have come from salt-tolerance studies of *Saccharomyces cerevisiae* cells [1]. In this microorganism, Pma1 $H^+$-ATPase regulates cytosolic pH by pumping protons through the plasma membrane out of the cell. The electrochemical gradient of protons created by Pma1 activity drives the secondary active transport of nutrients in symport with $H^+$ [2,3] and also controls the accumulation of essential $K^+$ ions. Potassium is actively taken up by the highly specific Trk1 and Trk2 proteins [4–6]. The efflux of potassium is mediated by the voltage-gated $K^+$-specific channel Tok1 [7] and moreover, the surplus $K^+$ is exported by two active systems, Ena $Na^+(K^+)$-ATPase [8] and Nha1 $Na^+(K^+)/H^+$ antiporter [9]. The balance achieved by $K^+$ and $H^+$ influx and efflux sets the steady state level of the plasma-membrane potential ($\Delta\Psi$; [10–12]).

Similarly, an electrochemical potential difference is maintained across the organellar membranes and it serves to energize the transport processes between the cytosol and organellar lumens. In yeast cells, it is mainly created by the activity of vacuolar Vma $H^+$-ATPase [13] and by redox pumps located in the mitochondrial inner membrane [14]. Three intracellular $K^+(Na^+)/H^+$ antiporters with different localizations have been described and characterized in *S. cerevisiae* cells so far. Nhx1p in the late endosomal/prevacuolar membranes [15], Kha1p in the membranes of the Golgi apparatus [16,17] and the third one, Vnx1p, has been reported to target vacuolar [18] and endoplasmic-reticulum [19] membranes. An antiport mechanism ensuring the physiologically highly important $K^+$ exchange for $H^+$ in the inner mitochondrial membrane was proposed as early as the early 60's [20]. The machinery controlling this electroneutral antiport is most probably a multi-subunit complex, but its molecular identity remains obscure [21,22].

Most attention has been focused on $K^+$, $Na^+$ and $H^+$ homeostases in yeast, and only very little on $Cl^-$ transport and its regulation. Despite the almost negligible $Cl^-$ fluxes in *S. cerevisiae* cells [23] and the failure to identify a plasma-membrane high-affinity $Cl^-$ transporter at the molecular level, there is evidence for the physiological relevance of $Cl^-$ fluxes. Yeast cells shifted to a low $Cl^-$ environment activate a sensing system in

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http://dx.doi.org/10.1016/j.bbamem.2012.09.019

which the product of *YHL008c* is a likely component [24]. It is known that $Cl^-$ moves via Gef1p, a member of the $Cl^-$ channel (CLC) family, into subcellular compartments such as Golgi and prevacuolar vesicles [25–27], and this channel might also be active at the plasma membrane [28]. Jennings and Cui (2008) showed that a significant fraction of $Cl^-$ is sequestered into the vacuole and prevacuolar vesicles in a Vma-ATPase-dependent manner. The importance of $Cl^-$ accumulation into these vesicles lies in the neutralization of protons pumped by Vma $H^+$-ATPase [29,30] and in copper assembly for the oxidase Fet3, an enzyme necessary for high-affinity iron uptake [31]. Another gene encoding a putative $Cl^-$ transporter, *YBR235w*, was found during the sequencing of chromosome II. Despite the Ybr235 protein's similarity with mammalian electroneutral cation-chloride cotransporters (CCC; [32]), neither the deletion nor the overexpression of the *YBR235w* gene affects the intracellular $Cl^-$ distribution [24].

CCCs (cation–chloride cotransporters; TC 2.A.30) are secondary active transporters that mediate the movement of $Cl^-$ tightly coupled to that of $K^+$ and/or $Na^+$ through the plasma membrane of eukaryotic cells [33]. In terms of function, three main classes of the CCC family can be distinguished; $Na^+$-coupled chloride cotransporters, $K^+$-coupled chloride cotransporters and a group of CCC proteins with unknown function [34,35]. In mammals, CCCs are expressed in the plasma membranes of many types of cells, and in humans, point mutations in genes encoding $K^+$–$Cl^-$ or $Na^+$–$K^+$–$2Cl^-$ cotransporters contribute to various diseases, e.g. Bartter's and Gitelman's syndrome [35].

To determine the function of Ybr235p in yeast cells we used a salt-sensitive strain lacking the main $Na^+$ and $K^+$ plasma-membrane efflux systems (*ena1-5Δ nha1Δ*) and thus having a higher cytosolic cation content. This approach enabled us to describe the impact of *YBR235w* deletion on cell growth under salt stress and on vacuolar morphology upon rapid osmotic changes. We show that Ybr235p, which we designated vacuolar protein homologous to CCC family 1 (Vhc1), is a vacuolar membrane protein that plays a role in $K^+$ homeostasis, probably as an electroneutral $K^+$–$Cl^-$ cotransporter.

## 2. Material and methods

### 2.1. Strains and media

The yeast strains used in this study were constructed by homologous recombination using the Cre-*loxP* system [36] and are listed in Table 1. Cells were grown either in rich YPD medium (1% yeast extract, 2% peptone, 2% glucose; 15 μg/ml adenine were added for W303-1A strain and its derivatives) or in various synthetic minimal media for different purposes: YNB (Difco; 0.67% yeast nitrogen base, 2% glucose) for the cultivation of cells bearing a plasmid, YNB-F (ForMedium™; 0.17% yeast nitrogen base without ammonium sulfate and potassium, 0.4% ammonium sulfate, 2% glucose, KCl at desired final concentration; buffered to pH 5.8 with ammonium hydroxide solution) for salt stress, drug sensitivity/tolerance and $K^+$ limitation tests, and YNB-pH (MP Biomedicals; 0.67% yeast nitrogen base without riboflavin and folic acid, 2% glucose) for the estimation of cytosolic pH. Synthetic minimal media were supplemented with the appropriate auxotrophic requirements. Solid media were prepared by adding 2% agar.

The *Escherichia coli* strain DH5α was used as a host for plasmids and was grown in Luria-Bertani (LB) broth with 100 μg/ml ampicillin at 37 °C.

**Table 1**
Yeast strains used in this work.

| Strain | Genotype | Source or reference |
|---|---|---|
| BY4741 | *MATa his3Δ1 leu2Δ met15Δ ura3Δ* | EUROSCARF |
| BYT9 | BY4741 *vhc1Δ::loxP* | This work |
| BYT12 | BY4741 *trk1Δ::loxP trk2Δ::loxP* | [69] |
| BYT128 | BY4741 *trk1Δ::loxP trk2Δ::loxP vnx1Δ::loxP* | This work |
| BYT129 | BY4741 *trk1Δ::loxP trk2Δ::loxP vhc1Δ::loxP* | This work |
| BYT1289 | BY4741 *trk1Δ::loxP trk2Δ::loxP vnx1Δ::loxP vhc1Δ::loxP* | This work |
| BYT45 | BY4741 *ena1-5Δ::loxP nha1Δ::loxP* | [12] |
| BYT458 | BY4741 *ena1-5Δ::loxP nha1Δ::loxP vnx1Δ::loxP* | This work |
| BYT459 | BY4741 *ena1-5Δ::loxP nha1Δ::loxP vhc1Δ::loxP* | This work |
| BYT4589 | BY4741 *ena1-5Δ::loxP nha1Δ::loxP vnx1Δ::loxP vhc1Δ::loxP* | This work |
| W303-1A | *MATa ade2-1 ura3-1 his3-11/15 trp1-1 leu2-3/112 can1-100* | [70] |
| WYBR | W303-1A *vhc1Δ::loxP* | This work |
| BW31a | W303-1A *ena1-4Δ::HIS3 nha1Δ::LEU2* | [71] |
| BWYBR | W303-1A *ena1-4Δ::HIS3 nha1Δ::LEU2 vhc1Δ::loxP* | This work |

### 2.2. DNA manipulation

Standard protocols for nucleic acid manipulations, yeast and *E. coli* transformations were carried out as described previously [37,38]. The *YBR235w* ORF plus 360 bp upstream was amplified from genomic DNA of *S. cerevisiae* BY4741 by PCR with Phusion™ DNA Polymerase (Finnzymes) and the oligonucleotides listed in Table 2. The amplified DNA fragment was cloned by homologous recombination either to the multi-copy pGRU1 (NCBI, Accession No. AJ249649) to tag its 3′ end with the GFP sequence (pVHC1-GFP) or YEp352 [39] resulting in pVHC1, respectively. The correct insertion was verified by restriction analysis and sequencing.

### 2.3. Salt and drug sensitivity tests

To estimate the growth capacities of yeast strains in the presence of salts or drugs, cells were grown overnight in YPD or YNB (for strains carrying a plasmid) media to saturation and washed twice with sterile distilled water. All cell suspensions were adjusted to an $OD_{600}$ of 0.2 and tenfold serial dilutions were prepared. 3 μl aliquots of each sample were spotted onto YNB-F media supplemented with high concentrations of salts (1100, 1200 and 1300 mM KCl or 500 and 700 mM NaCl). To test the sensitivity to nigericin, cells were spotted onto YNB-F solid media with 10 μg/ml nigericin (Sigma). For $K^+$-limiting experiments, yeast strains were grown overnight in YNB-F medium containing 50 mM KCl. Samples were washed and prepared as described above and spotted on YNB-F media supplemented with 2, 5, 10, 15, 25 and 50 mM KCl. Plates were incubated at 30 °C for 3–4 days. All experiments were repeated at least three times.

### 2.4. Tests of cell survival in YPD containing 2 M sorbitol

YPD-cultures of exponentially growing cells were diluted 1:1 with fresh YPD supplemented with 4 M sorbitol, and the $OD_{600}$ of cultures was followed in 30-min intervals over an 8-h period. At the same time intervals, aliquots of cultures were withdrawn, appropriately diluted and plated on YPD plates. The number of colonies (cfu) was estimated after 2 days of growth at 30 °C.

### 2.5. Assessment of relative $\Delta\Psi$ values

Relative values of plasma-membrane potential were measured with the fluorescent dye diS-$C_3(3)$ (3,3′-dipropylthiacarbocyanide iodide; Sigma) as described previously [12]. Briefly, cells were cultured in YNB-F medium containing 50 mM KCl overnight, washed twice with $K^+$-free YNB-F medium and incubated in a fresh sample of the same medium for 3 h. $K^+$-starved cells were then washed with sterile distilled water and twice with 10 mM $Na_2HPO_4$ (pH 6.0, adjusted with citric acid). Yeast suspensions were adjusted with the same buffer to an $OD_{600}$ of 0.2 and the potentiometric probe was added (0.2 μM final concentration). Fluorescence was measured using an ISS PC1 spectrofluorometer. The experiment was repeated at least three times and standard deviations calculated.

**Table 2**
A list of oligonucleotides used in this study.

| Name | Sequence |
|---|---|
| *Gene deletion oligonucleotides* | |
| YBR235W-kan-F | 5′-GCCAGCCAATTCTCTTTAAGAATAGGCCTTTCTTGTAGGATT ttcgtacgctgcaggtcgac-3′ |
| YBR235W-kan-R | 5′CTATATGTAGTAAATAAGTTCTGGGGAAATGCATAACGATATATGTGgcataggccactagtggatctg-3′ |
| VNX1-kan-F | 5′GTAAATGAAAGTGAAATAACTGCTAGCTAGAAGAGCGGTAAGCAGCttcgtacgctgcaggtcgac-3′ |
| VNX1-kan-R | 5′GAAAAATTGGTAGGTATCCAGGTGAAAAGCGGGGACAGTTGCTTTCgcataggccactagtggatctg-3′ |
| *Diagnostic oligonucleotides — target gene* | |
| YBR235W-UP | 5′-GGCAAGGCGCGGGCGGGTGAC-3′ |
| YBR235W-1R | 5′-CGTGCCAATGCCCTCTTGG-3′ |
| YBR235W-1 F | 5′-GAGACCCAATCGTGTTTGAT-3′ |
| YBR235W-DR | 5′-GCATCATATTTCTATACCATG-3′ |
| VNX1-UP | 5′-GCAGCGTAGCGAATGAATG-3′ |
| VNX1-1R | 5′-GTGTTGTTGCTGCTATGTACG-3′ |
| VNX1-1 F | 5′-GTAGCGTTATCAATGGAAATCG-3′ |
| VNX1-DR | 5′-GCGCTACCAAGCCACCCAAG-3′ |
| *Diagnostic oligonucleotides – deletion cassettes* | |
| KANX-R1 | 5′-ctctggggcgcatcgggc-3′ |
| KANX-F1 | 5′-catttgatgctcgatga-3′ |
| *Oligonucleotides for gene cloning into plasmids by homologous recombination* | |
| YBR235W-GFP-F[a] | 5′cgcaaaccgcctctccccgcgcgttggccgattcattcccCCAGCTTGTAGACGG CAACAACGG-3′ |
| YBR235W-GFP-R[a] | 5′-taaagctccggagcttgcatgcctgcaggtcgactctagaTGCAGTAGTTACTGTC ATGGTTTGC-3′ |
| YBR235W-YEp-F[b] | 5′-agcggataacaatttcacacagaaacagctatgaccatgCCAGCTTGTAGACGGC AACAACGG-3′ |
| YBR235W-YEp-R[b] | 5′-cggccagtgccaagcttgcatgcctgcaggtcgactctagCTATAATGCAGTAGT TACTGTCATGGTTTGC-3′ |

Region homologous to the *YBR235* ORF, upper-case letters.
[a] Region homologous to the pGRU1 plasmid, lower-case letters.
[b] Region homologous to the YEp352 plasmid, lower-case letters.

### 2.6. Cytosolic pH measurement

Cytosolic pH changes were monitored using pHluorin (a pH-sensitive ratiometric GFP; [40]) as described previously [15,41]. To estimate the intracellular pH, yeast strains transformed with a plasmid for pHluorin expression were grown to $OD_{600}$ 0.6–0.8 in YNB-pH medium containing all appropriate supplements with the exception of uracil. All experiments were repeated at least three times and standard deviations calculated.

### 2.7. Fluorescence and confocal microscopy

Cells harboring GFP-tagged Vhc1p were observed in an Olympus AX70 fluorescence microscope equipped with a U-MWB filter and also visualized using Nomarski differential-interference contrast (DIC) optics. Cells were viewed with a 100× oil immersion objective and images captured with an Olympus DP70 digital camera. For visualizing of vacuole morphology changes resulting from the osmotic shifts, cells were stained with FM4-64 [*N*-(3-triethylammoniumpropyl)-4-(p-diethylaminophenylhexatrienyl pyridinium dibromide; Molecular Probes] as described previously [42]). After staining, cells were resuspended either in fresh YPD (control), water (hypoosmotic stress) or YPD supplemented with 2 M sorbitol (hyperosmotic stress). Cells were examined immediately by confocal microscopy using a Leica DM IRE2 Laser Confocal Microscope System equipped with DIC optics. All images were captured at 63× magnification and with LCS software. For each independent experiment and time point indicated, over 250 cells were captured for the quantification of vacuolar morphological changes.

### 2.8. Data analysis

The accession numbers of the CCC proteins used in this study are as follows. Electroneutral cotransporters from *Homo sapiens*: KCC3, NP_005126.1; KCC1, NP_001139433.1; KCC4, NP_006589.2; KCC2, NP_001128243.1; NCC, NP_000330.2; NKCC1, NP_001037.1; NKCC2, NP_000329.2; CCC6, NP_064631.2. Putative electroneutral cotransporter from *S. cerevisiae*: Ybr235, NP_009794.1. ClustalW was used for protein sequence alignments [43]. *ScVHC1* orthologues were identified using the Genolevures database [44]. Phylogenetic analysis was performed via the Neighbor-Joining method using MEGA 4 software [45]. The statistical reliability of the phylogenetic trees was tested by bootstrap analysis with 1000 replicates. Moreover, genetic distances were calculated with the Poisson correction for proteins. The complete deletion option was used for handling gaps or missing data from alignments. Potential *N*-glycosylation sites were analyzed using NetNGlyc 1.0 Server (http://www.cbs.dtu.dk/services/NetNGlyc/).

## 3. Results

### 3.1. Ybr235p is highly homologous to human electroneutral cation–$Cl^-$ cotransporters

The *S. cerevisiae* genome contains only one ORF (*YBR235w*) annotated as a putative cation–$Cl^-$ cotransporter. The ORF encodes a protein of 1120 amino acid residues, with 28–32% sequence identity with human CCCs. Fig. S1 shows a full-length sequence alignment of Ybr235p and one example of each of the three functional classes of human CCCs (namely: NKCC1, KCC1 and CIP1 (CCC6)). This alignment shows a central, relatively well conserved, hydrophobic domain and hydrophilic N- and C-termini that differ both in their length and composition. The membrane part consists of 12 transmembrane (TM) segments according to the Kyte-Doolittle prediction [46]; however, the TMHMM 2.0 [47] and PredictProtein [48] topology analysis servers suggest a 10-TM topology model for Ybr235p. It is worth noting that the yeast protein has a much longer C-terminus than its human homologues (Fig. S1).

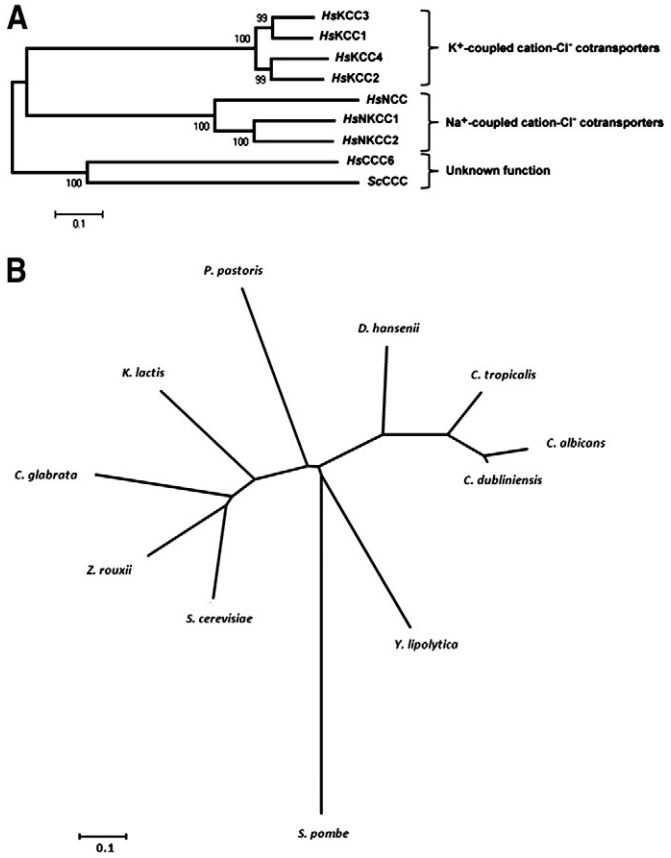


**Fig. 1.** Phylogenetic analysis of human and yeast CCCs. A. A phylogenetic tree of representative human CCC proteins and *S. cerevisiae* Ybr235 was created with the programs ClustalW and MEGA 4, respectively. The accession numbers of the CCC proteins are as follows. Human cotransporters: *Hs*KCC3, AF314956; *Hs*KCC1, NM_005072; *Hs*KCC4, NM_006598; *Hs*KCC2, AF208159; *Hs*NCC, NM_000339; *Hs*NKCC1, NM_000329; *Hs*NKCC2, NM_000338; *Hs*CCC6 (CIP1), NM_020246. Yeast Ybr235 protein: *Sc*CCC, NP_009794. B. Phylogenetic three of yeast CCC transporters. The phylogenetic three was drawn from yeast CCC protein sequences using MEGA 4 software. Accession numbers are listed in Table S1.

The three CCC groups of proteins differ in the number and position of their *N*-linked glycosylation sites [35]. $K^+$-coupled chloride cotransporters and CIP1 (CCC6) possess a large *N*-glycosylated extracellular loop between TM5 and TM6, while with $Na^+$-coupled chloride cotransporters, it is between TM7 and TM8. Ybr235p does not have any potential *N*-linked glycosylation sites in these two regions. On the other hand, three putative and Ybr235-specific *N*-glycosylation sites are present in its very long C-terminal hydrophilic tail (Fig. S1), similar to the yeast late endosomal/prevacuolar Nhx1 antiporter, a member of the NHE family, whose C-terminus is glycosylated at two asparagine residues [49], whereas none of its mammalian NHE homologues possesses an *N*-glycosylation site on their C-terminal tail.

As illustrated in Fig. 1A, the yeast Ybr235p is the most related to human CCC6 (CIP1), a member of the CCC family, which has been only partially characterized. This protein has been reported to be a CCC-interacting protein which regulates the activity of NKCC1 and KCC2 by forming the heterodimers NKCC1-CIP1 [50] and KCC2-CIP1 [51], respectively.

Genes highly homologous to *S. cerevisiae YBR235w* probably exist in all yeast species (Fig. 1B). A detailed search for the presence of CCC orthologous sequences revealed 88 putative CCCs in yeasts and filamentous fungi (their accession numbers, protein length, sequence identity values and conserved domains are summarized in Table S1). Until now, none of the fungal CCCs had been studied and experimentally characterized. Therefore, elucidating the subcellular localization of the Ybr235p and characterizing the phenotypes of *YBR235w* deletion/overexpression in *S. cerevisiae* cells were pivotal to understanding the physiological role of yeast CCC proteins.

### 3.2. Ybr235 is a vacuolar membrane protein

To examine the localization of CCC yeast homologue, we constructed a plasmid in which the sequence of green fluorescent protein (GFP) was fused to the 3′ end of *YBR235w*. Ybr235-GFPp localized exclusively intracellularly, and the GFP fluorescence signal colocalized with FM4-64 (Fig. 2), which selectively incorporates into the vacuolar membranes and those of the vesicles of the endocytic pathway [42]. Due to the localization of the *YBR235w* gene's product and also its significant homology to the CCC protein family, we propose to refer to the yeast *YBR235w* gene as *VHC1* (vacuolar protein homologous to CCC family 1).

### 3.3. Deletion of the VHC1 gene exacerbates the $K^+$ sensitivity phenotype of strains lacking major $Na^+$ and $K^+$ efflux systems

The similarity of the *VHC1* gene product to CCCs suggests that Vhc1p might participate in cellular ion homeostasis. To determine its physiological function in yeast cells, the chromosomal copy of *VHC1* was deleted in various strains. First, single BYT9 and WYBR mutants (*vhc1*Δ in the BY4741 and W303-1A genetic backgrounds, respectively; Table 1) were constructed and tested for their ability to grow in YNB-F medium supplemented with either high $K^+$ or $Na^+$ salts. This single deletion produced no detectable changes in the salt tolerance of the two parental strains (Fig. 3A; shown only for the BY4741 genetic background). But when the effect of the *VHC1* deletion was tested in a salt-sensitive strain lacking both plasma-membrane Ena $Na^+(K^+)$-ATPase and the Nha1 $Na^+(K^+)/H^+$ antiporter, a strong phenotype in the presence of high KCl was observed (Fig. 3A; shown for the BY4741-derived mutants). After 2 days of incubation at 30 °C in the presence of 1.1 M KCl, the growth of the triple BYT459 mutant lacking *VHC1* (*ena1-5*Δ *nha1*Δ *vhc1*Δ) was significantly impaired compared to the growth of the BYT45 (*ena1-5*Δ *nha1*Δ) strain (Fig. 3A). Interestingly, the BYT459 (*ena1-5*Δ *nha1*Δ *vhc1*Δ) strain grew the same as BYT45 (*ena1-5*Δ *nha1*Δ) when cells were propagated on YNB-F plates with a high concentration of a $Na^+$ salt. A similar increased KCl sensitivity and no change in NaCl sensitivity were observed for strains with the W303-1A genetic background, i.e. BWYBR (*ena1-4*Δ *nha1*Δ *vhc1*Δ) vs. BW31a (*ena1-4*Δ *nha1*Δ) cells (data not shown). This increased sensitivity to high KCl concentrations may result from the hyperpolarization of the plasma membrane leading to a higher intracellular potassium concentration. The relative values of plasma-membrane potential of the two wild types (BY4741 and W303-1A) and relevant mutants were estimated and the data obtained (summarized in Fig. S2) showed only a marginal hyperpolarization of strains lacking *VHC1*. All these results suggested the involvement of Ybr235p in the regulation of intracellular potassium homeostasis and not in the detoxification of sodium cations by their sequestration in vacuoles.

To confirm this hypothesis, the effect of *VHC1* overexpression on yeast cells exposed to high $K^+$ salts was tested. The *VHC1* gene with its own promoter was cloned into a multi-copy plasmid and expressed in both the BYT45 (*ena1-4*Δ *nha1*Δ) and BYT459 (*ena1-4*Δ *nha1*Δ *vhc1*Δ) mutant strains. The growth of the resulting transformants was tested on YNB-F media containing 1300 mM KCl (Fig. 3B). As expected, overexpression of the *VHC1* gene gave BYT459 cells a higher tolerance to external KCl. In addition, the presence of extra copies of *VHC1* also led to an improvement in the KCl tolerance of BYT45 (*ena1-4*Δ *nha1*Δ) cells, i.e. cells with a chromosomal copy of the *VHC1* gene (Fig. 3B). These results provide molecular evidence that vacuoles play an important role in yeast salt tolerance. Similar results, i.e. increased tolerance to high external KCl, were obtained when the cells were transformed

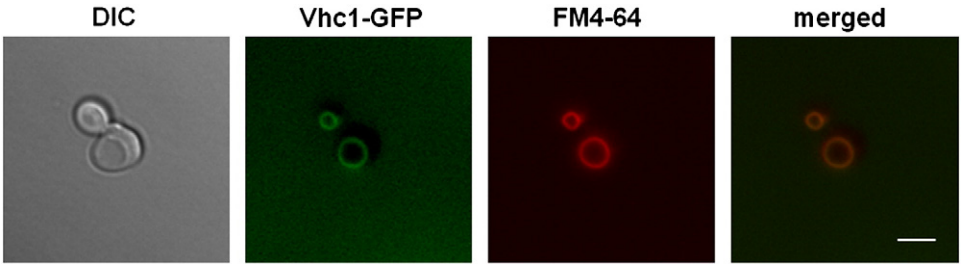


**Fig. 2.** *Sc*Ybr235p is localized in the vacuolar membrane. Fluorescent images demonstrating the colocalization of Vhc1(Ybr235)-GFP (green) with FM4-64 (red) giving a yellow merged signal are shown. DIC, differential interference contrast. The scale bar is 5 μm.

with pVHC1-GFP and produced C-terminally GFP-tagged Vhc1 protein (not shown). Taken together, we propose that the lack of a *VHC1* gene in cells without active plasma-membrane cation exporters leads to an increase in cytosolic $K^+$ levels and in contrast, its overexpression enhances the sequestration of cytosolic $K^+$ into yeast vacuoles.

### *3.4. Deletion of VHC1 affects the vacuolar morphology upon hyperosmotic stress*

The localization of Vhc1p in the vacuolar membrane led us to inspect the vacuolar morphology of mutant cells lacking the *VHC1* gene. Since the importance of inorganic ions in osmoregulation is well known, we asked whether Vhc1p, being a candidate for a $K^+$–$Cl^-$ cotransporter in the yeast vacuole, might be involved in the regulation of this process. To investigate this, cells were stained with the FM4-64 dye. In exponentially growing BY4741 cells under standard conditions (YPD), vacuoles are usually visible as 1–3 smaller round structures. The same morphological appearance was observed in the wild-type BY4741, in the single BYT9 mutant (*vhc1*Δ; Fig. 4), in BYT45 (*ena1-5*Δ *nha1*Δ) and in BYT459 (*ena1-5*Δ *nha1*Δ *vhc1*Δ) cells (Fig. 5A). However, when the cells were exposed to a hypo- or hyperosmotic environment, their vacuoles changed in size and shape (as a consequence of the uptake or release of water and solutes). Under hypoosmotic conditions, yeast vacuoles fuse into one large round organelle [52], whereas hyperosmotic stress induces their fragmentation [53–55]. In our experiments, the transfer of FM4-64 stained cells from YPD to water, i.e. to hypoosmotic conditions, in all tested strains resulted in the fusion of small vacuoles into one large vacuole (not shown). Upon hyperosmotic shock (transfer of cells from YPD to YPD + 2 M sorbitol), significant differences were observed between strains carrying and lacking the *VHC1* gene. The vacuoles of BYT45 (*ena1-5*Δ *nha1*Δ) and wild-type BY4741 cells rapidly and totally shrank (shown for BYT45 in Fig. 5A). Almost no round structures were present in these strains. All their vacuoles already had a shrunken appearance within the first minute of perfusion with YPD medium supplemented with 2 M sorbitol (Fig. 5A). In BYT459 (*ena1-5*Δ *nha1*Δ *vhc1*Δ) cells, vacuoles shrank to a much smaller extent than in BYT45 (*ena1-5*Δ *nha1*Δ) cells and several round vacuoles were still detectable even after 25 min of a hyperosmotic stress (only ~25% of the cell population harbored dramatically shrunken vacuoles; Fig. 5B). Long-term exposure of BYT45 (*ena1-5*Δ *nha1*Δ) cells to high sorbitol restored normal vacuolar morphology. More than 70% of cells with *VHC1* harbored 1–3 enlarged vacuoles after 90 min of stress while the vacuolar morphology of the BYT459 (*ena1-5*Δ *nha1*Δ *vhc1*Δ) triple mutant was the same during the whole experiment (Fig. 5B). The same results were obtained with mutant cells in the W303-1A genetic background (data not shown). Monitoring the $OD_{600}$ of cultures after the transfer of cells to hyperosmotic conditions (YPD supplemented with 2 M sorbitol) revealed that the BYT45 (*ena1-5*Δ *nha1*Δ) culture restored its growth after 180 min and the BYT459 (*ena1-5*Δ *nha1*Δ *vhc1*Δ) cells only started to divide after 270 min. A noticeable difference was observed when the survival rate (cfu) of cells in YPD with 2 M sorbitol was tested. Only approx. 2% of BYT459 (*ena1-5*Δ *nha1*Δ *vhc1*Δ) cells were able to form colonies after 180 min in YPD with 2 M sorbitol, whereas 10% of BYT45 (*ena1-5*Δ *nha1*Δ) cells survived the same

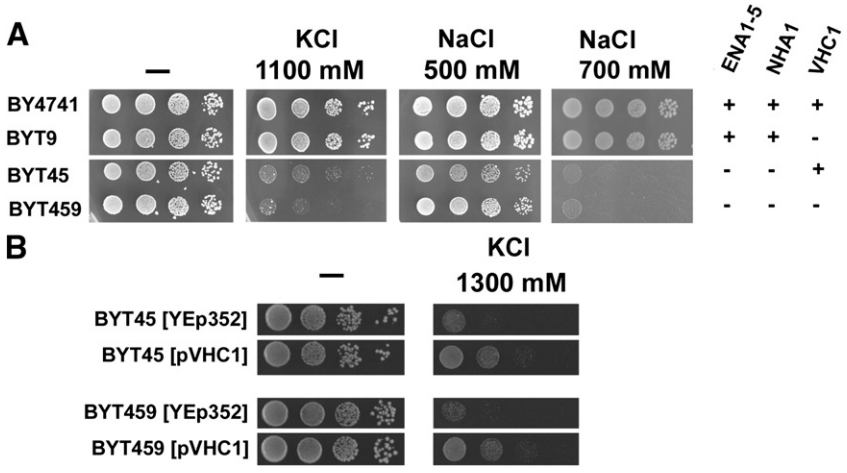


**Fig. 3.** *VHC1* is important for the yeast tolerance of high external KCl concentrations. A. BY4741 strain and its derivatives BYT9 (*vhc1*Δ), BYT45 (*ena1-5*Δ *nha1*Δ) and BYT459 (*ena1-5*Δ *nha1*Δ *vhc1*Δ) were tested for salt tolerance on solid YNB-F media supplemented with indicated amounts of KCl and NaCl. B. Salt tolerance of BYT45 (*ena1-5*Δ *nha1*Δ) and BYT459 (*ena1-5*Δ *nha1*Δ *vhc1*Δ) cells expressing *VHC1* from YEp352 plasmid (pVHC1) was determined on solid YNB-F media supplemented with 1300 mM KCl. Cells transformed with the empty YEp352 served as controls.

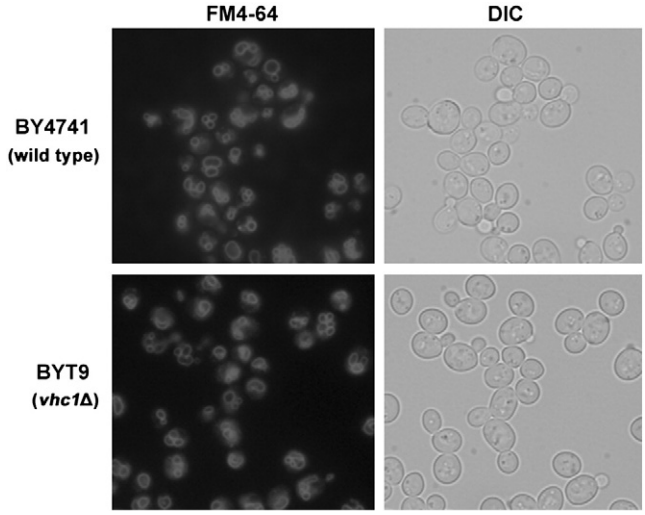


**Fig. 4.** Deletion of *VHC1* does not change vacuolar morphology under standard growth conditions. Fluorescent (left) and Nomarski/DIC (right) images of wild-type BY4741 and BYT9 (*vhc1*Δ) cells grown in YPD medium and labeled with FM4-64 are shown.

conditions. It is worth noting that a significant difference was observed 30 min after cell transfer to hyperosmotic conditions (colonies formed by 40% and 60% of BYT459 and BYT45 cells, respectively, cf. Supplementary Table 2). These results, obtained under non-salt hyperosmotic conditions, confirmed that Vhc1p has a general role in osmoregulation.

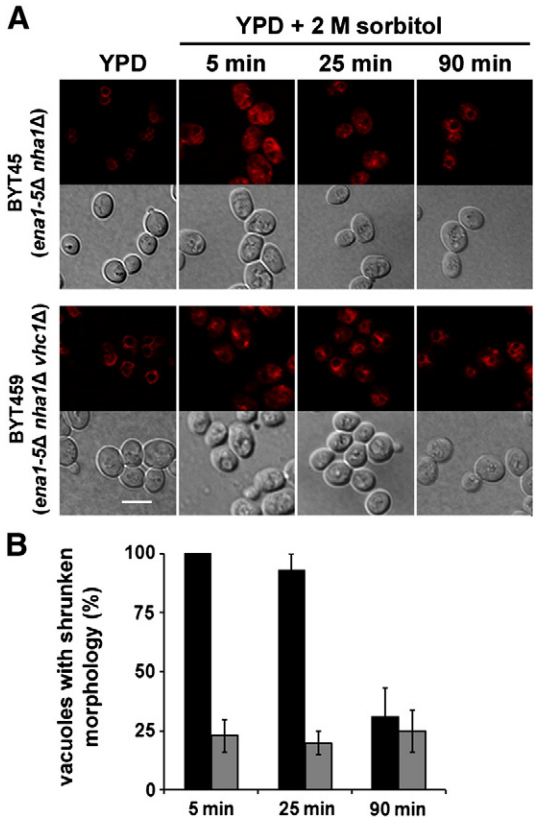


**Fig. 5.** Absence of *VHC1* gene affects changes in vacuolar morphology upon hyperosmotic stress. A. BYT45 (*ena1-5*Δ *nha1*Δ) and BYT459 (*ena1-5*Δ *nha1*Δ *vhc1*Δ) cells were grown in YPD, stained with FM4-64 fluorescent dye, then transferred either to fresh YPD or YPD medium containing 2 M sorbitol, and visualized by confocal microscopy. Fluorescent (upper) and Nomarski/DIC (bottom) images of stressed cells were taken at the indicated time points after transfer to the stress condition. The scale bar is 5 μm. B. Quantification of cells with totally shrunken vacuoles (in %) during incubation in YPD medium containing 2 M sorbitol. At least 250 cells were examined for each strain (BYT45, black columns and BYT459, gray columns) and each time point.

### 3.5. Vhc1 and Vnx1 proteins work as organellar $K^+$ suppliers with a different mode of action

So far, the only characterized potassium transporter in yeast vacuolar membranes has been the Vnx1 $Na^+(K^+)/H^+$ exchanger [18]. Because of the possible functional overlap between the Vhc1 and Vnx1 transporters, we constructed two series of strains with deletions of the two genes for vacuolar transporters (*vnx1*Δ and/or *vhc1*Δ) and genes for the plasma-membrane potassium uptake (*trk1*Δ *trk2*Δ; BYT12) and efflux systems (*ena1-5*Δ *nha1*Δ; BYT45; see Table 1). First, we compared the sensitivity of the BYT45 (*ena1-5*Δ *nha1*Δ) derivatives BYT458 (*ena1-5*Δ *nha1*Δ *vnx1*Δ), BYT459 (*ena1-5*Δ *nha1*Δ *vhc1*Δ) and BYT4589 (*ena1-5*Δ *nha1*Δ *vnx1*Δ *vhc1*Δ) strains to high concentrations of alkali–metal–cation salts. As expected, the BYT458 (*ena1-5*Δ *nha1*Δ *vnx1*Δ) strain lacking the Vnx1 antiporter was more sensitive to high NaCl than its parental BYT45 (*ena1-5*Δ *nha1*Δ). However, the combination of *vnx1*Δ and *vhc1*Δ had no additive effect on cell salt sensitivity. The growth inhibition of BYT4589 (*ena1-5*Δ *nha1*Δ *vnx1*Δ *vhc1*Δ) cells on high KCl and NaCl plates was the same as that of the BYT458 (*ena1-5*Δ *nha1*Δ *vnx1*Δ) and BYT459 (*ena1-5*Δ *nha1*Δ *vhc1*Δ) strains (results not shown).

We next tested the contribution of Vhc1 and Vnx1 transporters to the maintenance of cytosol $K^+$ homeostasis in a $K^+$-uptake deficient strain. These cells (BYT12; *trk1*Δ *trk2*Δ) are unable to grow on YNB-F plates containing low amounts of $K^+$ [12]. The growth of wild-type BY4741, BYT12 (*trk1*Δ *trk2*Δ), BYT128 (*trk1*Δ *trk2*Δ *vnx1*Δ), BYT129 (*trk1*Δ *trk2*Δ *vhc1*Δ) and BYT1289 (*trk1*Δ *trk2*Δ *vnx1*Δ *vhc1*Δ) cells on YNB-F media with various KCl concentrations was estimated. Plates with a KCl concentration (50 mM) sufficient for growth of the BYT12 (*trk1*Δ *trk2*Δ) strain served as a positive control. As shown in Fig. 6, the presence of 10 mM KCl in YNB-F medium was not sufficient for the growth of the BYT12 mutant. Surprisingly, the additional deletion of either *VHC1* or *VNX1* led to a growth improvement (Fig. 6), most probably due to an increase in cytosolic $K^+$ content as a consequence of the diminished sequestration of potassium into the vacuole. Importantly, the simultaneous deletion of both the *VNX1* and *VHC1* genes did not have an additional effect. The growth rates of BYT128 (*trk1*Δ *trk2*Δ *vnx1*Δ), BYT129 (*trk1*Δ *trk2*Δ *vhc1*Δ) and BYT1289 (*trk1*Δ *trk2*Δ *vnx1*Δ *vhc1*Δ) strains in the presence of various limiting KCl concentrations were the same. Strains lacking the Trk1 and Trk2 transporters were also reported to be sensitive to low external pH [56]. This phenotype appears to be a consequence of their significantly decreased intracellular pH [12]. Vnx1p has also been proposed to play a role in cellular pH homeostasis [18]. Taking into account all of these facts, we decided to measure the intracellular pH of the wild-type BY4741, BYT12 (*trk1*Δ *trk2*Δ) and BYT12-derived strains lacking either the Vnx1 or

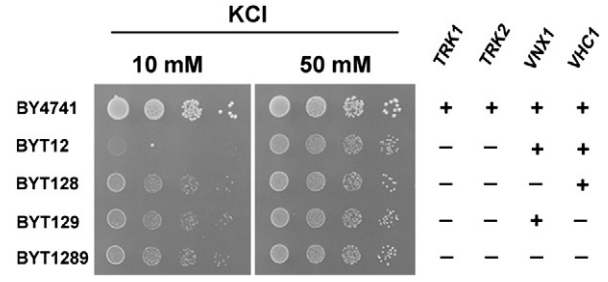


**Fig. 6.** Lack of vacuolar Vhc1 and Vnx1 potassium transporters improves the growth of *trk1*Δ *trk2*Δ cells under $K^+$-limiting growth conditions. Growth of wild-type BY4741 and its derivatives BYT12 (*trk1*Δ *trk2*Δ), BYT128 (*trk1*Δ *trk2*Δ *vnx1*Δ), BYT129 (*trk1*Δ *trk2*Δ *vhc1*Δ) and BYT1289 (*trk1*Δ *trk2*Δ *vnx1*Δ *vhc1*Δ) was estimated on solid YNB-F plates containing either 10 or 50 mM KCl.

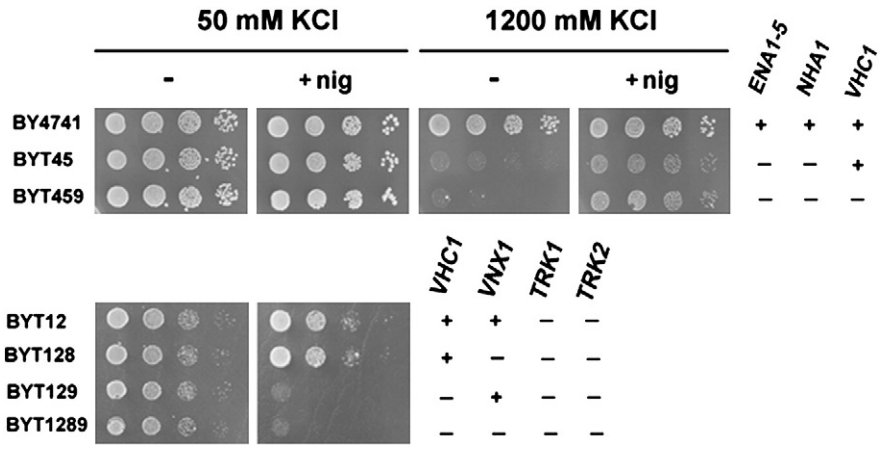


**Fig. 7.** Nigericin influences the phenotypes of strains lacking the *VHC1* gene. The effect of the presence of nigericin on the growth of the BY4741 strain and its derivatives BYT45 (*ena1-5*Δ *nha1*Δ), BYT459 (*ena1-5*Δ *nha1*Δ *vhc1*Δ), BYT12 (*trk1*Δ *trk2*Δ), BYT128 (*trk1*Δ *trk2*Δ *vnx1*Δ), BYT129 (*trk1*Δ *trk2*Δ *vhc1*Δ) and BYT1289 (*trk1*Δ *trk2*Δ *vnx1*Δ *vhc1*Δ) was followed in a drop test on solid YNB-F media containing either 50 or 1200 mM KCl, and with or without the addition of nigericin (10 μg/ml).

Vhc1 transporters. As shown in Fig. S3, BYT12 (*trk1*Δ *trk2*Δ) cells had a significantly lower pH than the wild-type cells, and BYT128 (*trk1*Δ *trk2*Δ *vnx1*Δ) cells lacking Vnx1p exhibited even lower pH values. However, deletion of the *VHC1* gene in the BYT12 genetic background (BYT129; *trk1*Δ *trk2*Δ *vhc1*Δ) had no effect on the intracellular pH.

### 3.6. Deletion of VHC1 gene produces nigericin-sensitive phenotype in yeast cells lacking main $K^+$ uptake transporters

Nigericin is an electroneutral ionophore which catalyzes a $K^+$ exchange for $H^+$ across biological membranes [57] and can substitute for the activity of potassium transporters. For example, in yeast cells, it has been shown to operate as a chemical suppressor for a lack of $K^+/H^+$ exchange activity in the inner mitochondrial membrane [21,58]. As we show here that Vhc1p contributes to $K^+$ homeostasis and probably also to the transport of this cation into *S. cerevisiae* vacuoles, we decided to test whether *vhc1*Δ may have an effect on the nigericin sensitivity/tolerance of cells under various external or intracellular $K^+$ conditions. The growth of the BYT9 strain with a single *VHC1* deletion was not affected by the presence of the drug (not shown). Also, the combination of *vhc1*Δ and *ena1-5*Δ *nha1*Δ mutations (BYT459 strain) had no phenotype on YNB-F plates containing 50 mM KCl and 10 μg/ml nigericin (Fig. 7). On the other hand, the addition of nigericin to YNB-F medium with 1200 mM KCl suppressed the $K^+$ sensitivity of BYT459 (*ena1-5*Δ *nha1*Δ *vhc1*Δ) cells as well as its parental BYT45 (*ena1-5*Δ *nha1*Δ) strain. This simple experiment illustrates how the nigericin $K^+/H^+$ exchange activity can restore the growth of mutant cells unable to actively export potassium out of cells by sequestering of surplus $K^+$ into intracellular organelles (most likely to the vacuole).

Unlike the BYT9 (*vhc1*Δ) and BYT459 (*ena1-5*Δ *nha1*Δ *vhc1*Δ) mutant strains, *vhc1*Δ in combination with the *trk1*Δ *trk2*Δ mutations (the BYT129 strain) led to a marked growth inhibition on YNB-F plates supplemented with 50 mM KCl and nigericin (Fig. 7). This phenotype was specific to the absence of Vhc1p. The deletion of *VNX1* had no effect on the growth of cells in the presence of KCl and nigericin (Fig. 7). These results, together with the internal pH measurements described above, make it less likely that Vhc1 and Vnx1 transporters contribute to vacuolar ion homeostasis in the same way.

## 4. Discussion

The *S. cerevisiae* genome only harbors one gene homologous to the family of eukaryotic cation–$Cl^-$ cotransporters, *YBR235w*. Homologous genes exist in the genomes of probably all yeasts and filamentous fungi (Table S1) but the function of their products has not yet been studied. Phylogenetically, the Ybr235 protein belongs to the functionally less characterized subclade of CCC proteins which also includes human CCC6 (CIP1), which is thought not to be a real transporter, but to interact with plasma-membrane cation–$Cl^-$ cotransporters from other subclades and regulate their activity [50,51]. As shown in Table S1, most fungal putative CCC proteins contain a conserved K–Cl cotransporter domain (TIGR00930 2a30/K–Cl cotransporter) and an amino-acid-permease domain (pfam00324/Aa_permease). The presence of an amino-acid-permease domain is not surprising as the CCC proteins are known to constitute a subfamily of the amino acid–polyamine–choline (APC) superfamily (cf. http://tcdb.org// database; [59]). The level of sequence similarity of CCC proteins with members of the well-characterized APC superfamily (members of which occur in bacteria, archea, fungi, plants and animals) suggests that the CCC family is an old protein family with roots in the prokaryotic kingdom [60]. This theory has been supported by the determination of the X-ray structure of a hydrophilic C-terminal domain of a CCC protein from the archea *Methanosarcina acetivorans* [61]. Likewise in eukaryotic CCC transporters, the archeal *Ma*CCC protein shares common structural features (a transmembrane transport domain followed by a regulatory C-terminal domain) and a dimerization of the *Ma*CCC has also been observed [61].

We have shown that the yeast member of this subclade is directly involved in the cell tolerance to KCl and it resides in vacuolar membranes. An analysis of the secondary structure of Ybr235p shows a typical membrane transporter with 10–12 transmembrane domains. Ybr235p is more likely to have 12 transmembrane domains, since this was experimentally found for the closely related cotransporter NKCC1 [62]. As shown in the alignment of CCC sequences (Fig. S1), the amino-acid residues conserved in mammalian members of the family and known to be important for their function [63,64], mainly in the region covering TM segments 2–3 and the connecting loop, are also well conserved in the yeast homologue. Another well-conserved region is located at the beginning of the hydrophilic C-termini of all CCCs (Fig. S1), and contains several candidate sites for protein kinases, for interactions with other regulatory proteins and between CCCs themselves [50,51].

While all human cation–chloride cotransporters characterized so far have been plasma membrane-associated proteins, our results showed the GFP-tagged Ybr235p to be located exclusively in the membranes of vacuoles. This finding is consistent with the results of a high-throughput analysis of the yeast vacuolar proteome that also identified the *YBR235w* product [65]. Taking into account its close relationship to the CCC family, Ybr235p is the most likely candidate for an alkali–

metal–cation–$Cl^-$ cotransporter in the vacuole(s) of *S. cerevisiae* cells and thus we propose to call it Vhc1. Our salt-tolerance studies, performed in two genetic backgrounds with identical results, strongly suggest that Vhc1p facilitates $K^+$–$Cl^-$ cotransport into the vacuole, which is the major storage site of $Cl^-$ [24] and $K^+$ [66,67] ions in yeast cells, and it plays a role in $K^+$ homeostasis by sequestering surplus cytosolic $K^+$ into vacuoles.

Another result supporting the $K^+$–$Cl^-$ cotransport activity of Vhc1p in yeast vacuoles is its role in vacuole volume control upon hyperosmotic shock. Analogously, human NKCC1 and $K^+$–$Cl^-$ cotransporters, such as KCC2 and KCC3, are known to regulate intracellular $Cl^-$ levels to maintain normal cell volume and their dysfunction has detrimental effects on neural cells (reviewed in [68]). It is worth noting that in human cells, hypertonicity induces the inward transport of $Cl^-$ (with $Na^+$ and $K^+$ ions) mediated by NKCC1 and water through the plasma membrane, thereby causing them to swell. In the opposite case, hypotonicity triggers the efflux of $K^+$ and $Cl^-$ ions out of cells via the KCC2 or KCC3 cotransporters resulting in the loss of water, thereby decreasing the cell volume. In this study we examined the contribution of Vhc1p to yeast general osmotolerance by inspecting the vacuolar morphology of *vhc1*Δ cells and their ability to survive after transfer to a medium containing a high concentration of a non-salt solute. Cells lacking Vhc1p shrank much less and survived the osmotic shock significantly worse than cells harboring the *VHC1* gene. The observed phenotypes are similar to those observed in the short-term response to hyperosmotic stress of cells lacking the *NHX1* gene encoding the prevacuolar/endosomal cation/proton antiporter [54]. Thus we speculate that the activity of both the Vhc1 and Nhx1 transporters contribute to the yeast cell adaptation to an unfavorable hyperosmotic environment by regulating the fluxes of ions and water between the cytosol and vacuolar lumen.

The function of Vhc1p in $Cl^-$ transport has not been tested directly in our study as it would be, purely for technical reasons, very difficult. In addition, earlier experiments including transport measurements showed that yeast cells harbor a mechanism which maintains a steady-state intracellular $Cl^-$ levels as neither a deletion nor an overexpression of the *VHC1* gene led to significant $Cl^-$ flux changes [24]. In the same work, another uncharacterized protein, Yhl008, has been proposed to work as a $Cl^-$ sensor, though its cellular localization remains unknown. Although the authors speculated that the accumulation of $Cl^-$ in the vacuole(s) and prevacuolar organelles could be mediated via Gef1p [24], their results clearly showed that none of the three proteins (Vhc1, Yhl008, Gef1) is a high-affinity $Cl^-$ transporter.

As Vhc1p is a vacuolar protein, this led us to compare the properties of yeast cells without this transporter and cells lacking another intracellular alkali–metal–cation transporter, Vnx1. Originally, the Vnx1 protein was reported to be vacuole-membrane-associated [18,65] but recently, in another study, it has been predominantly found in the membranes of the endoplasmic reticulum and only partially in Golgi, prevacuolar compartments and the vacuole [19]. Our comparative phenotypic studies in the genetic background of cells with abolished plasma-membrane $K^+$ uptake suggest that Vhc1p and Vnx1p contribute to a decrease in cytosolic potassium content with different modes of action. The absence of either of the two transporters improves the growth of *trk1*Δ *trk2*Δ cells on low potassium media (Fig. 6), probably due to the fact that less potassium is transported into the vacuoles, and thus its cytosolic content is higher and cells more easily reach the minimum $K^+$ concentration contributing to the cytosolic turgor necessary for cell growth and division [1]. However, the nigericin hypersensitivity was only observed in cells lacking the *VHC1* gene (Fig. 7), and on the contrary, only the deletion of *VNX1* resulted in a decrease in cytosolic pH (Fig. S3). Accordingly, we were unable to detect any effect of *VHC1* deletion on cell sensitivity to low or high pH (data not shown) whereas it was found in *vnx1*Δ mutants [18,19]. Likewise, *vhc1*Δ cells differ in their pH-related phenotypes from *nhx1*Δ cells [26]. Taken together, our results produced several lines of evidence that the Vhc1 cation–chloride cotransporter contributes to potassium homeostasis in the cytosol and vacuoles, but its mode of action is independent of pH unlike the activity of the Vnx1 and Nhx1 alkali–metal–cation/proton antiporters.

### 4.1. Conclusions

In conclusion, our findings indicate that the *S. cerevisiae VHC1* gene product is the first member of the CCC family found to function intracellularly. It is involved in cytosol/vacuole potassium transport and plays a role in vacuolar morphology control during the early phase of cell response to a hyperosmotic stress.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbamem.2012.09.019.

## Acknowledgements

We would like to acknowledge the contribution of our technician Pavla Herynkova to the construction of mutant deletion strains and for performing the sorbitol survival tests. We thank Iveta Fajnerova and Alexandr Cernavsky (Department of Biomathematics) for their help with the confocal microscopy experiments. This work is a part of the European Transnational Funding and Research Program – SysMO – Translucent 2 and was supported by grants from the Academy of Sciences CR (M20011090), GA AS CR (IAA500110801), MSMT (LC531 and COST OC10012), and Institutional Concept (AV0Z50110509).

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