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RESEARCH ARTICLE

K^+-specific importers Trk1 and Trk2 play different roles in Ca^{2+} homeostasis and signalling in *Saccharomyces cerevisiae* cells

Olga Zimmermannova[1,*,†], Kristina Felcmanova[1], Lenka Sacka[1], Anne-Sophie Colinet[2], Pierre Morsomme[2] and Hana Sychrova[1]

[1]Laboratory of Membrane Transport, Institute of Physiology of the Czech Academy of Sciences, Videnska 1083, Prague 4 - Krc, 142 20, Czech Republic and [2]Louvain Institute of Biomolecular Science and Technology, Université catholique de Louvain, B 1348, Louvain-la-Neuve, Belgium

*Corresponding author: Laboratory of Membrane Transport, Institute of Physiology CAS, Videnska 1083, Prague 4 - Krc, 142 20, Czech Republic. Tel: +420 241 062 557; Fax: +420 241 062 488; E-mail: olga.zimmermannova@fgu.cas.cz

One sentence summary: The plasma-membrane K^+-importer Trk1 is critical for the immediate Ca^{2+}-induced cell response to stresses and cell resistance to high extracellular calcium, whilst Trk2 influences steady-state intracellular Ca^{2+} levels.

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†Olga Zimmermannova, http://orcid.org/0000-0003-2538-4488

ABSTRACT

The maintenance of K^+ and Ca^{2+} homeostasis is crucial for many cellular functions. Potassium is accumulated in cells at high concentrations, while the cytosolic level of calcium, to ensure its signalling function, is kept at low levels and transiently increases in response to stresses. We examined Ca^{2+} homeostasis and Ca^{2+} signalling in *Saccharomyces cerevisiae* strains lacking plasma-membrane K^+ influx (Trk1 and Trk2) or efflux (Tok1, Nha1 and Ena1-5) systems. The lack of K^+ exporters slightly increased the cytosolic Ca^{2+}, but did not alter the Ca^{2+} tolerance or Ca^{2+}-stress response. In contrast, the K^+-importers Trk1 and Trk2 play important and distinct roles in the maintenance of Ca^{2+} homeostasis. The presence of Trk1 was vital mainly for the growth of cells in the presence of high extracellular Ca^{2+}, whilst the lack of Trk2 doubled steady-state intracellular Ca^{2+} levels. The absence of both K^+ importers highly increased the Ca^{2+} response to osmotic or $CaCl_2$ stresses and altered the balance between Ca^{2+} flux from external media and intracellular compartments. In addition, we found Trk2 to be important for the tolerance to high KCl and hygromycin B in cells growing on minimal media. All the data describe new interconnections between potassium and calcium homeostasis in *S. cerevisiae*.

Keywords: potassium; calcium; K^+-transporter; osmotic shock; Trk1; Trk2; yeast

ABBREVIATIONS

EGTA:	Ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid;
BAPTA:	1,2-bis(o-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid;
YNB:	Yeast nitrogen base;
YPD:	Yeast extract peptone dextrose;
SD:	Standard deviation;
aa:	Amino acid(s);
OD:	Optical density;
TMA^+:	Tetramethylammonium;
HygB:	Hygromycin B;

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INTRODUCTION

The budding yeast *Saccharomyces cerevisiae*, being a unicellular organism, has to continuously adapt to environmental changes. It has therefore developed intracellular signalling systems that identify these stimuli and implement cellular responses to counteract the stress. Potassium and calcium are among the essential cations that participate in cellular adaptation. Appropriate intracellular concentrations of K^+, Ca^{2+} and other cations are ensured by the coordinated activity of various transporters localized either in the plasma membrane or in membranes of intracellular organelles (Espeso 2016; Yenush 2016).

Saccharomyces cerevisiae can grow in the presence of a broad range of concentrations of external potassium (10 μM–2.5 M; Yenush 2016). In cells, potassium cations have to be accumulated at a relatively high concentration as they are important in cell bioenergetics and are required for the compensation of negative charges in many macromolecules, maintenance of cell volume, the cell cycle or for the cell response to osmotic shock (Arino, Ramos and Sychrova 2010). Intracellular K^+ content corresponds to the steady state between simultaneous influx and efflux across the plasma membrane, and the coordination of K^+ fluxes is necessary for the maintenance of potential across the plasma membrane and intracellular pH (Arino, Ramos and Sychrova 2019). In *S. cerevisiae*, the influx of K^+ is ensured by two K^+-specific transporters, Trk1 (Gaber, Styles and Fink 1988) and Trk2 (Ko and Gaber 1991). Trk1 is the prominent mediator of K^+ uptake (Bertl *et al.* 2003). Trk2 is less important for K^+ accumulation in exponentially growing cells (Petrezselyova, Ramos and Sychrova 2011), while it was found to be vital under specific stress conditions, e.g. anhydrobiosis (Borovikova *et al.* 2014) or under low K^+ and pH stress in the *sin3* genetic background (Michel *et al.* 2006). K^+ efflux is provided by three types of alkali–metal–cation transporters: Na^+, K^+/H^+ antiporter Nha1 (Banuelos *et al.* 1998), Na^+, K^+- ATPase Ena1-5 (Haro, Garciadeblas and Rodriguez-Navarro 1991) and K^+-specific channel Tok1 (Bertl *et al.* 1993). The absence of high-affinity potassium uptake (the *trk1*Δ *trk2*Δ mutant) results in plasma membrane hyperpolarization and, consequently, in a decrease in intracellular pH due to a downregulation of Pma1 H^+-ATPase, i.e. the main creator of plasma-membrane potential (Navarrete *et al.* 2010; Zimmermannova *et al.* 2015). This proves a tightly regulated cross-talk between K^+ and H^+ transporters at the plasma membrane (Arino, Ramos and Sychrova 2010). In addition, several already characterized systems transporting potassium cations are localized in the membranes of endosomes (Na^+, K^+/H^+ antiporter Nhx1; Nass, Cunningham and Rao 1997), the Golgi apparatus (K^+/H^+ antiporter Kha1; Maresova and Sychrova 2005), mitochondria (K^+/H^+ antiporter Mkh1; Froschauer, Nowikovsky and Schweyen 2005), or vacuoles (Na^+, K^+/H^+ antiporter Vnx1 (Cagnac *et al.* 2007), K^+-Cl^- co-transporter Vhc1 (Petrezselyova, Kinclova-Zimmermannova and Sychrova 2013)), where they ensure an import of K^+ into organelles and thus also contribute to establishing the appropriate potassium concentration inside the cells.

Saccharomyces cerevisiae also tolerates a wide range of extracellular concentrations of calcium, but, in contrast to K^+, the cytosolic concentration of Ca^{2+} is maintained at very low levels (Espeso 2016). In cells, calcium is employed in the regulation of a wide variety of processes such as cell-cycle progression, mating, protein processing, responses to osmotic stress, maintenance of intracellular pH or nutritional and metabolic signalling (Cyert and Philpott 2013; Thewes 2014; Espeso 2016; Park *et al.* 2019). Upon an environmental stress, e.g. an osmotic shock, the cytosolic Ca^{2+} level transiently increases via a sudden and massive cation influx from the external medium and/or internal stores (e.g. vacuole; Denis and Cyert 2002; Matsumoto *et al.* 2002). From the external medium, calcium can flow in either via a high-affinity influx system (HACS formed by proteins Cch1, Mid1 and Ecm7), and a less-defined low affinity Ca^{2+} influx system (LACS) consisting of various components belonging to the polarisome, proteins involved in actin polymerization or gene products required for cell fusion during mating (reviewed in Cyert and Philpott 2013; Liu *et al.* 2015). The product of the *ECM27* gene, which encodes a putative Na^+/Ca^{2+} exchanger, was also found to be responsible for Ca^{2+} influx from the extracellular space as well as release from intracellular stores during membrane stress (Klukovich and Courchesne 2016). Some additional Ca^{2+} influx pathways whose activation depends on growth conditions or hypotonic stress, but have not been characterized at the molecular level, were described biochemically (Groppi *et al.* 2011; D'Hooge *et al.* 2015; Rigamonti *et al.* 2015). An increased Ca^{2+} influx through the plasma membrane is inhibited by the plasma-membrane protein Rch1 (Zhao *et al.* 2016). The specific hypotonic-stress-induced calcium influx is negatively regulated by the ER protein Cls2 and involves the protein product of the *FLC2* gene (Rigamonti *et al.* 2015). As for Ca^{2+} efflux from cells, Hong *et al.* detected a H^+ coupled exchange of Ca^{2+} in plasma membrane vesicles via biochemical methods (Hong *et al.* 2013), but the gene encoding this system has not been identified yet.

Calcium could also be released to the cytosol from vacuolar stocks via the mechanosensitive channel Yvc1 (Palmer *et al.* 2001; Zhou *et al.* 2003). After the release, the low basal cytosolic calcium concentration is rapidly restored by specific Ca^{2+} transporters localized in the vacuolar membrane (Ca^{2+}-ATPase Pmc1; Cunningham and Fink 1994), Ca^{2+}/H^+ antiporter Vcx1 (Cunningham and Fink 1996) or in other organelles (Ca^{2+}-ATPase Pmr1 in Golgi (Antebi and Fink 1992) or putative Ca^{2+}-ATPase Spf1/Cod1 in the endoplasmic reticulum (Cronin, Rao and Hampton 2002)). It was recently found that, in response to various stresses, the activation of plasma-membrane Cch1 and vacuolar Yvc1 requires glutathionylation and depends on the redox state in the cell (Chandel, Das and Bachhawat 2016; Chandel and Bachhawat 2017).

An elevated concentration of calcium in the cytosol (induced by a stress) is immediately sensed by various Ca^{2+}-binding proteins, including calmodulin—a major target of Ca^{2+}. Calmodulin, in its Ca^{2+}-bound form, binds to and activates protein kinases and calcineurin, a conserved serine/threonine phosphatase (Cyert 2003). Calcineurin dephosphorylates a range of protein targets, including the Crz1 transcription factor. The dephosphorylation of Crz1 results in its translocation to the nucleus, where it influences the expression of various genes, including those encoding ion pumps (Pmc1, Pmr1 and Ena1) (Cyert and Philpott 2013; Liu *et al.* 2015; Park *et al.* 2019).

The disruption of calcineurin signalling also influences potassium uptake and increases potassium requirements (Casado *et al.* 2010). The absence of calcineurin affects high-affinity K^+ uptake mediated by Trk1 under normal growth conditions as well as in the presence of salt stress (Mendoza *et al.* 1994; Casado *et al.* 2010). The mechanism of Trk1 activity regulation employs calcineurin-dependent up-regulation of the gene encoding Hal5 kinase, which is thought to modulate Trk1 trafficking and its affinity state (Casado *et al.* 2010). All these data indicate that calcium (and calcineurin) exert a complex influence on cation homeostasis, and that there is an interplay between calcium and potassium homeostasis in *S. cerevisiae*.

In this work, we examined in detail Ca^{2+} homeostasis and Ca^{2+} signalling in yeast cells with disturbed K^+ homeostasis. We determined the Ca^{2+} levels and monitored cytoplasmic changes in the concentration of Ca^{2+} cations in various *S. cerevisiae* strains lacking K^+ influx (Trk1 and Trk2) or efflux (Tok1, Nha1 and Ena1-5) systems. The lack of K^+ exporters had a relatively minor effect on the calcium response to hyperosmotic stress. On the other hand, we found that the K^+-importers Trk1 and Trk2 play different roles in the maintenance of Ca^{2+} homeostasis and signalling. While the presence of Trk1 was vital mainly for the growth of cells in the presence of high extracellular Ca^{2+}, the lack of Trk2 resulted in an elevated total amount of Ca^{2+} in non-stressed cells. The absence of both K^+ importers significantly disturbed Ca^{2+} stress response and signalling in cells. In summary, our data describe new interconnections between potassium and calcium homeostasis in *S. cerevisiae* cells.

METHODS

Yeast strains and growth media

The yeast strains used in this study are listed in Table 1. Yeast cultures were routinely grown in YPD (Formedium, Hunstanton) or in YNB media (Difco, Franklin Lakes) at 30°C. YNB-based media (referred here as YNB-NH_4Cl) contained 0.2% YNB without amino acids and ammonium sulphate (with a $CaCl_2$ content of approx. 1.8 mM), 0.35% ammonium chloride, 2% glucose and 100 mM KCl (the addition of KCl was necessary to support the growth of strains lacking K^+ importers Trk1, 2). Ammonium chloride was used instead of ammonium sulphate as a nitrogen source to avoid precipitation when $CaCl_2$ was added. YNB-F plates with a defined amount of potassium contained 0.17% YNB-F without amino acids and ammonium sulphate (Formedium), 0.4% ammonium chloride, 2% glucose. The pH of YNB-F was adjusted to 5.8 with ammonium hydroxide solution. The mixture of required auxotrophic supplements (mixtures OMM or OMM-ura for transformed cells) from a 50x-concentrated sterile stock solution prepared according to (Hansch o *et al.* 2012) was added after media sterilization. $CaCl_2$ was added from 3 M $CaCl_2$ stock solution after sterilization. Inositol at a final concentration of 10 μg/mL was added after sterilization when cells were growing in liquid YNB media. Solid media were prepared by adding 2, 2.5 or 3% (w/v) agar (media without salts, gradient plates or gradient plates supplemented with KCl or NaCl, respectively).

Growth assays

For the drop tests, cells pregrown on YNB-NH_4Cl + 100 mM KCl plates were suspended in sterile water to OD_{600} = 0.6 (Spekol 211, Carl Zeiss, Jena). Serial 10-fold dilutions of cell suspensions were prepared and spotted on YNB-NH_4Cl + 100 mM KCl supplemented with increasing concentrations of $CaCl_2$. Gradient plates were prepared by pouring two media layers differing in their compositions as described previously (Maresova and Sychrova 2005). First sloping layers containing the highest amount of tested compound ($CaCl_2$, KCl, NaCl and tetramethylammonium (TMA^+; Sigma) or hygromycin B (Sigma, Darmstadt), as indicated) were covered with a second layer of media of the same composition, only without the salt or cationic drug. When gradient plates with increasing K^+ concentration were prepared, the first layer was composed of only YNB-F (containing 15 μM KCl and residual K^+ from agar), and the second layer was the same YNB-F media supplemented with 50 mM KCl. For the assessment of growth on gradient plates, fresh cells suspensions of each strain of the same OD_{600} = 0.3 were spotted on plates. The more tolerant to the tested compound a particular strain was, the more drops in the line were able to grow. All plates were incubated at 30°C. Each experiment was repeated three times and representative pictures are shown.

For the growth curves, measurements in liquid YNB-NH_4Cl + 100 mM KCl media without or supplemented with 0.2 M $CaCl_2$, cells were inoculated to OD_{600} = 0.02 in 100 μL of media in a 96-well microplate and cell growth was monitored in an ELx808 Absorbance Microplate Reader (BioTek, Winooski) as described previously (Maresova and Sychrova 2007). A total of eight technical replicates were measured for each strain and set of conditions in individual experiments. The experiment was repeated three times with similar results and the values shown are the mean ± SD of the ratio of OD_{600} in media with $CaCl_2$ vs. OD_{600} in media without $CaCl_2$ reached in 24 h.

Whole-cell Ca^{2+} content

Yeast cells pre-cultured in YNB-NH_4Cl + 100 mM KCl medium at 30°C for 1 day were inoculated into the same fresh medium (60 mL) and grown over-night at 30°C to a final OD_{600} = 0.5. Cells (50 mL) were collected by vacuum filtration using membrane filters (Glass Microfiber filter Whatman; cat. no. 1825-025) and washed successively twice with 2 mL of cold 1 mM EGTA (pH 8.0) and twice with 2 mL of cold H_2O. Cells were then extracted overnight in solution containing 10 mM $MgCl_2$ and 0.2 M HCl and the calcium content in extracts was analysed by inductively-coupled-plasma-mass-optical-emission spectrometry (ICP-OES Avio-500, Perkin Elmer, Waltham) and the intracellular Ca^{2+} contents were calculated based on the cell dry weight (according to the experimentally-obtained equation OD_{600} = 1.0 = 0.85 mg dry wt) of samples and the dilution factor. Whole-cell Ca^{2+} content values are expressed as millimoles per kg dry weight of cells. Data shown are the average of three independent repetitions ± SD.

Changes in cytosolic Ca^{2+} content

Cytosolic calcium concentration in response to NaCl-osmotic shock or to the addition of $CaCl_2$ was monitored using an aequorin-based assay (Matsumoto *et al.* 2002; Demaegd *et al.* 2013). The strains listed in Table 1 were transformed with pEVP11-AEQ (Batiza, Schulz and Masson 1996) to express aequorin in their cytosols. Cells were pre-cultured in YNB-NH_4Cl + 100 mM KCl medium at 30°C for 1 day, inoculated into the same fresh medium supplemented with 10 μM coelenterazine H (Sigma-Aldrich, Darmstad) and incubated at 30°C in black 15 mL tubes (HS4428, Heathrow Scientific, Vernon Hills) overnight. When cells reached OD_{600} ~ 0.5, 100 μL of suspension was transferred to the 96-well white Nunclon Delta microplate (cat. no. 136 101, Thermo Fisher Scientifc, Waltham and the luminescence was recorded every 2 s for 5 min using a Cytation 3 reader (Biotek) to obtain the basic level of luminescence. Then, a high sodium or calcium shock was performed by the addition of 50 μL of 4 M NaCl or 0.4 M $CaCl_2$ to reach the final concentration of 1.3 M NaCl or 0.133 M $CaCl_2$, respectively. The signal was measured for the next 15 min until it again reached the original (basic) level of luminescence. Cells were then lysed by adding 200 μL of a solution containing 20% EtOH + 2 M $CaCl_2$, and the luminescence signal was recorded for an additional 60 min. The time course of changes in cytosolic concentration

Table 1. *Saccharomyces cerevisiae* strains used in the study.

Strain	Genotype	Reference
BY4741	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	EUROSCARF
Derivatives of BY4741:		
BYT1	*trk1Δ::loxP*	Navarrete *et al.* (2010)
BYT2	*trk2Δ::loxP*	Petrezselyova, Ramos and Sychrova (2011)
BYT12	*trk1Δ::loxP trk2Δ::loxP*	Navarrete *et al.* (2010)
BYT345	*tok1Δ::loxP nha1Δ::loxP ena1-5Δ::loxP*	Zahradka and Sychrova (2012)
BYT12345	*trk1Δ::loxP trk2Δ::loxP tok1Δ::loxP nha1Δ::loxP ena1-5Δ::loxP*	Navarrete *et al.* (2010)

were then calculated using the equation in (Allen, Blinks and Prendergast 1977). When the effect of extracellular EGTA was estimated, 100 μL of cell suspension was mixed with 10 μL of 100 mM EGTA (final concentration 9.1 mM) immediately prior to luminescence monitoring. Each experiment was repeated at least four times for each strain, and conditions and changes in cytosolic Ca^{2+} are shown either as representative curves, average curves ± SD, or as average values reached at particular points of the curves ± SD.

Statistics

Data were analysed either with Microsoft Excel software 2016 or SigmaPlot 11, and *P*-values were calculated using the two-tailed Student's T-test or One-way ANOVA (Dunnett's method), respectively.

RESULTS

The intracellular concentration of potassium cations in *S. cerevisiae* cells growing in a laboratory media with sufficient amount of K^+ and negligible amount of sodium salts ranges from 200 to 300 mM (Arino, Ramos and Sychrova 2010). Under these conditions, potassium is accumulated predominantly in vacuoles, the cytosol and nucleus (45%, 15% and 29% of total K^+, respectively; Herrera *et al.* 2013). As was mentioned above, the cytosolic concentration of Ca^{2+} is much lower (50–200 nM), and increases only transiently to activate downstream events under specific conditions, such as exposure to environmental stresses (Cyert and Philpott 2013). To examine a possible interconnection between K^+ and Ca^{2+} homeostasis, we compared the Ca^{2+}-related phenotypes of wild-type BY4741 cells to isogenic strains lacking the K^+ importers Trk1 and/or Trk2 (strains BYT1, BYT2 and BYT12, respectively), a strain which lacked all three plasma-membrane K^+ exporters – K^+ channel Tok1, Na^+, K^+/H^+ antiporter Nha1 and Na^+, K^+-ATPases Ena1-5 (strain BYT345) and a strain deficient in all five plasma-membrane K^+ influx and efflux systems (BYT12345; Table 1).

Lack of Trk2 decreases cell tolerance to high KCl and hygromycin B

At first, we verified the phenotypes related to the presence of particular mutation(s) and the corresponding disturbed K^+ and Na^+ homeostasis of all six strains in one drop test on gradient plates based on minimal media (YNB and YNB-F) with ammonium chloride as a nitrogen source, which was then used for testing Ca^{2+}-related parameters (cf. below and Methods). Solid media contained gradually increasing concentrations of KCl or NaCl, cationic drugs (tetramethylammonium (TMA^+), hygromycin B (HygB)), or in the case of YNB-F, decreasing concentrations of K^+ (Fig. 1). No differences in growth among strains were observed on the control plate supplemented with an optimal (100 mM) concentration of KCl to support the growth of all strains (Fig. 1). It is well known that the lack of K^+ importers Trk1 and Trk2 cause insufficient K^+ accumulation (Navarrete *et al.* 2010; Petrezselyova, Ramos and Sychrova 2011). Correspondingly, in the drop test shown in YNB-F in Fig. 1, BYT1 (*trk1Δ*) grew slower in the presence of low K^+ than wild-type cells. In general, the lack of the *TRK2* gene has a minor effect on growth in the presence of low K^+ concentrations than the absence of *TRK1* (Petrezselyova, Ramos and Sychrova 2011), and its participation in K^+ accumulation is only evident in the double-mutated strain *trk1Δ trk2Δ* (BYT12), which is more sensitive to low K^+ than the *trk1Δ* (BYT1) strain (Fig. 1). On the other hand, high external KCl can suppresses a deficiency in K^+ influx, and thus BYT1 and also BYT12 cells grew the same and as well as the wild type on gradient plates with high KCl (Fig. 1). Surprisingly, and to our knowledge for the first time, we observed that the lack of only Trk2 resulted in a higher sensitivity to elevated KCl than wild-type cells (Fig. 1).

Plasma-membrane hyperpolarization, resulting from the absence of Trk-mediated K^+ uptake, also causes an increased non-specific uptake of monovalent cations (Madrid *et al.* 1998). Correspondingly, both strains (BYT1(*trk1Δ*) and BYT2 (*trk2Δ*)) were more sensitive to high external NaCl than wild-type cells, and the double-mutated *trk1Δ trk2Δ* cells (BYT12) exhibited even lower NaCl tolerance (Fig. 1). BYT1 cells were also more sensitive to tetramethylammonium (TMA^+), which is a cationic drug entering cells proportionally to the plasma-membrane potential (Fig. 1). Even higher sensitivity to TMA^+ of BYT12 reflected the participation of Trk2 in this phenotype (Fig. 1), though it was not visible in the single *trk2Δ* mutant (BYT2; Fig. 1). All three strains (BYT1, BYT2 and BYT12) also grew slower than wild-type cells in the presence of another cationic drug—hygromycin B, but, unexpectedly, under these conditions, the sensitivity of BYT2 cells, which only lack Trk2, was higher than that of cells lacking Trk1 (Fig. 1).

As expected, the lack of the K^+ (Na^+) efflux systems Tok1, Nha1 and Ena (the BYT345 strain) resulted in a higher sensitivity of the strain to an elevated concentration of KCl and NaCl (due to the inability of cells to export surplus K^+ or Na^+ from cells), but had no effect on growth in the presence of low K^+ or cationic drugs (Fig. 1). Strain BYT12345 lacking all five transporters exhibited the highest sensitivity to all the stress conditions tested (Fig. 1), and in agreement with previous results, BYT12345 cells exhibited slightly better growth on low K^+ than

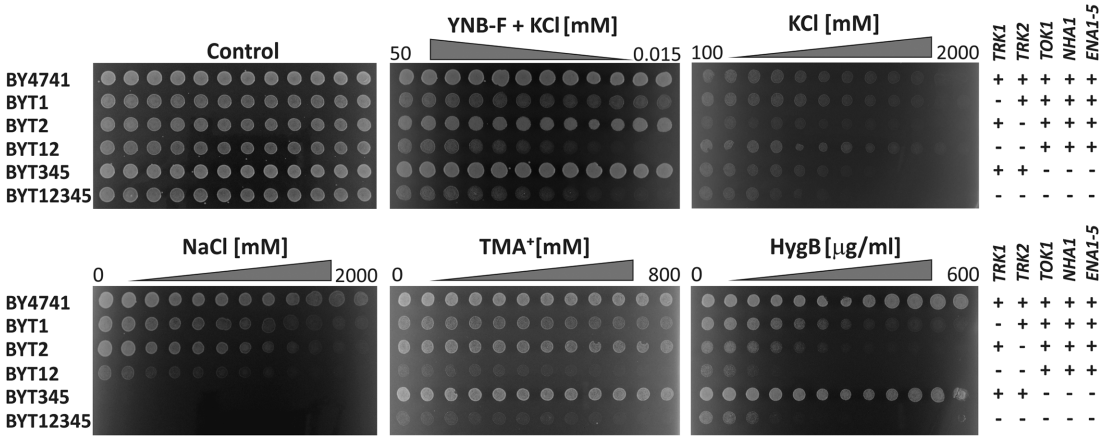

Figure 1. Cation sensitivity of yeast strains lacking various combinations of K^+ transporters. Drop lines of equal initial OD_{600} were spotted on YNB-NH_4Cl + 100 mM KCl medium (Control), on YNB-NH_4Cl + 100 mM KCl plates containing gradients of NaCl, KCl, tetramethylammonium (TMA^+) or hygromycin B (Hyg B), or on YNB-F medium with a gradient of KCl as indicated. Cells were incubated for 1 day (Control, YNB-F, TMA^+, hygromycin B) or 2 days (NaCl, KCl) at 30°C. The drop lines correspond to the BY4741, BYT1 (*trk1*Δ), BYT2 (*trk2*Δ), BYT12 (*trk1*Δ *trk2*Δ), BYT345 (*tok1*Δ *nha1*Δ *ena1*-5Δ) or BYT12345 (*trk1*Δ *trk2*Δ *tok1*Δ *nha1*Δ *ena1*-5Δ) strains as indicated. The drop test was repeated three times and representative pictures are shown.

BYT12, as these cells do not lose too much internal K^+ necessary for cell growth via K^+ exporters (Zahradka and Sychrova 2012).

The drop test confirmed that the absence of these transporters results in a similar disturbance of monovalent-cation homeostasis in the presence of two different ammonium salts (ammonium chloride (to avoid Ca^{2+} precipitation in our next experiments) and ammonium sulphate (Navarrete *et al.* 2010; Petrezselyova, Ramos and Sychrova 2011; Zahradka and Sychrova 2012). In addition, two new phenotypes related to *trk2* deletion were revealed. Further, we examined whether the lack of these monovalent cation transporters also influences the homeostasis of divalent Ca^{2+} cations.

Lack of K^+ importers affects Ca^{2+} sensitivity

To prove the hypothesized connection between the presence/activity of K^+ transporters and Ca^{2+} homeostasis, we tested the growth of the same set of strains in media supplemented with $CaCl_2$. Similar results were obtained in liquid (Fig. 2A) as well as on solid (Fig. 2B) media. The presence of 0.2 M $CaCl_2$ in media affected the growth of wild-type cells, cells lacking the Trk2 transporter (strain BYT2) or K^+ efflux systems (strain BYT345) in a similar manner (Fig. 2A and not shown, respectively). On the other hand, cells deficient in the Trk1 system (BYT1) grew significantly slower than wild-type cells in the presence of 0.2 M $CaCl_2$ (Fig. 2A). Cells without both Trk systems (BYT12) grew slightly worse than the BYT1 strain, indicating that the lack of Trk2 also to some extent decreases the ability of yeast cells to grow in the presence of elevated extracellular $CaCl_2$ (Fig. 2A). This was confirmed on solid media. When the growth of the six strains was tested on plates supplemented with a higher concentration of $CaCl_2$ (0.4 or 0.6 M; Fig. 2B), we observed that not only the lack of Trk1, but also the absence of Trk2 transporter (BYT2 cells) resulted in a slower growth than the wild-type strain (Fig. 2B; 0.6 M $CaCl_2$). Similarly, as in liquid media, high $CaCl_2$ did not influence the growth of cells lacking K^+ exporters (Fig. 2A and B). The deletion of genes for all five transporters caused the same sensitivity of cells to the presence of $CaCl_2$ as the deletion of Trk importers, both in liquid media as well as on plates (Fig. 2; BYT12 and BYT12345), which confirmed that K^+ exporters are not important for the cell tolerance to high extracellular $CaCl_2$.

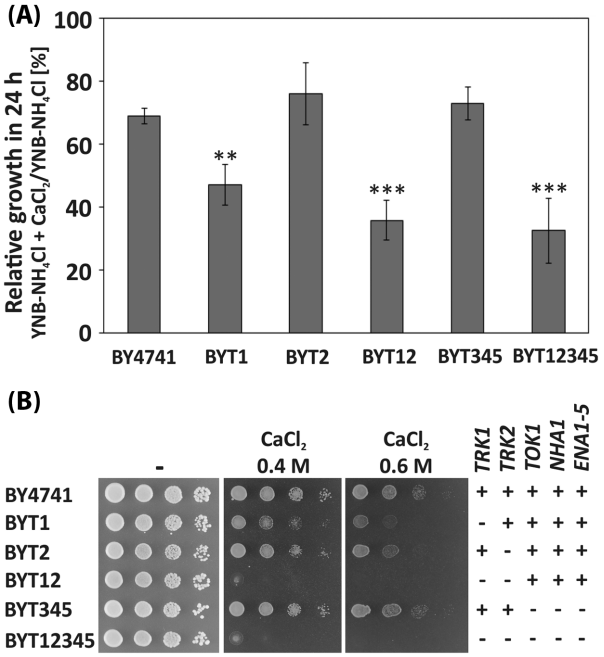

Figure 2. Lack of K^+ importers Trk1 and Trk2 increases cell Ca^{2+} sensitivity. The BY4741 wild type and its five derivatives lacking various cation transporters were **(A)** inoculated into liquid YNB-NH_4Cl + 100 mM KCl medium without or supplemented with 0.2 M $CaCl_2$, and the optical density of cultures was measured within 30 h of growth at 30°C. The ratio of OD_{600} reached in 24 h in media with $CaCl_2$ vs. media without $CaCl_2$ is plotted. The growth of the particular strain in media without $CaCl_2$ was regarded as 100%. Mean values ± SD obtained in three independent experiments are shown. Significant differences from the growth of wild-type cells are indicated with asterisks (** $P < 0.01$; *** $P < 0.001$). **(B)** Cells were spotted on YNB-NH_4Cl + 100 mM KCl medium supplemented with $CaCl_2$ as indicated. Pictures correspond to plates incubated at 30°C for 2 days. The drop test was repeated three times and representative pictures are shown.

Lack of Trk2 increases intracellular Ca^{2+} content

Then, we estimated the total intracellular concentration of Ca^{2+} in all six strains grown in YNB-NH_4Cl + 100 mM KCl (Fig. 3). Surprisingly, Ca^{2+}-sensitive cells without Trk1 (BYT1) contained a similar amount of total Ca^{2+} to wild-type cells (BY4741) or

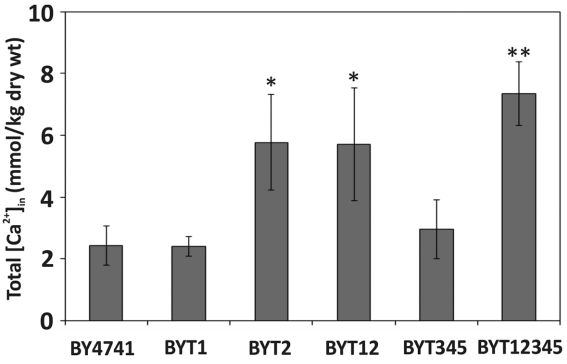

Figure 3. Lack of K^+ importer Trk2 increases the intracellular amount of Ca^{2+} in the absence of stress. BY4741 wild type and its derivative strains lacking various cation transporters (BYT1 (*trk1*Δ), BYT2 (*trk2*Δ), BYT12 (*trk1*Δ *trk2*Δ), BYT345 (*tok1*Δ *nha1*Δ *ena1-5*Δ) or BYT12345 (*trk1*Δ *trk2*Δ *tok1*Δ *nha1*Δ *ena1-5*Δ)) were grown in YNB-NH_4Cl + 100 mM KCl to $OD_{600} = 0.5$ and the total intracellular Ca^{2+} was estimated as described in Material and *Methods*. The calculated mean values ± SD obtained in three independent experiments are plotted. Significant differences from wild-type cells are indicated with asterisks (* $P < 0.05$; ** $P < 0.01$).

cells lacking cation efflux systems (BYT345 cells; Fig. 3). On the other hand, BYT2 cells with a deleted Trk2 transporter contained almost double Ca^{2+} level of the wild-type strain (Fig. 3). Similarly elevated total intracellular Ca^{2+} was also observed in two other strains harbouring the *TRK2* deletion (Fig. 3; BYT12 and BYT12345).

These results indicated that calcium homeostasis is interconnected more to K^+ import systems Trk1 and/or Trk2 than to K^+ exporters. While Trk1 is mainly important for the growth of cells in the presence of increased extracellular Ca^{2+}, the absence of Trk2 increases the total amount of Ca^{2+} accumulated in cells (grown under non-stressed conditions).

Lack of K^+-uptake systems increases Ca^{2+} response to salt stress

The exposure of yeast cells to a high concentration of NaCl triggers a sudden increase in cytosolic Ca^{2+} concentration that results from Ca^{2+} influx through plasma-membrane transporters and/or release from intracellular organelles (Denis and Cyert 2002; Matsumoto *et al.* 2002; Rigamonti *et al.* 2015). The resting calcium level is then restored by the reabsorption of Ca^{2+} mainly into the vacuole (Denis and Cyert 2002). To test whether the lack of any K^+ transporter affects this process, we employed an aequorin-based assay (Denis and Cyert 2002) and followed the calcium response to salt stress (1.33 M NaCl) in the same set of strains, which expressed the Ca^{2+} sensor aequorin in the cytosol (cf. Methods). The assay enabled us to directly monitor changes in the cytosolic concentration of Ca^{2+} before and after the shock and to determine three parameters related to Ca^{2+} response to salt stress for each strain: the basal level of free Ca^{2+} in the cytosol, the size of the peak of cytosolic Ca^{2+} concentration after the addition of NaCl and the rapidity of reabsorption of Ca^{2+} from the cytosol to intracellular compartments to reach the basal level again. The addition of NaCl clearly triggered Ca^{2+} signalling in all cells (Fig. 4B). A characteristic curve of changes in free cytosolic Ca^{2+} in BY4741 cells during the experiment with highlighted particular phases/parameters is shown in Fig. 4A. Figure 4B shows representative curves obtained for the wild type and five mutated strains. To highlight differences in the shapes of curves among strains immediately after the NaCl shock, only the curves obtained within the first 18 min of the experiment are plotted (i.e. before the cell lysis cf. Fig. 4A and B).

As for the basal cytosolic Ca^{2+} concentration, strains lacking K^+ importers maintained a significantly lower (BYT1 (*trk1*Δ)) or higher (BYT2 (*trk2*Δ)) basal Ca^{2+} concentration in the cytosol at the beginning of the experiment compared to the wild-type cells (Fig. 4B and C). The lack of three K^+ exporters (BYT345 strain) also resulted in a significantly increased level of the basal cytosolic Ca^{2+} level compared to the wild-type cells (Fig. 4B and C). In contrast, when the genes encoding both Trk transporters were deleted either alone (strain BYT12) or in combination with genes for exporters (strain BYT12345), cells exhibited similar basal levels of cytosolic Ca^{2+} to the BY4741 strain (Fig. 4B and C).

After exposure to NaCl stress, the cytosolic level of Ca^{2+} increased sharply in all strains, nevertheless the cytosolic Ca^{2+}-concentration peaks varied between strains (Fig. 4B). To compare the response, we not only followed the curves, but we also calculated the increase in Ca^{2+} cytosolic concentration after the addition of NaCl (i.e. the Ca^{2+} concentration reached at the peak minus the basal Ca^{2+} level), and all the obtained data were statistically analysed (Fig. 4D). Strain BYT2, which lacks the Trk2 transporter, exhibited a similar shape of response to NaCl stress to wild-type cells (Fig. 4B), and the increase in cytosolic Ca^{2+} concentration was not significantly different from wild-type cells (Fig. 4D). The peak of cytosolic Ca^{2+} concentration after NaCl addition was the smallest in the BYT345 strain, which lacks all K^+ exporters (Fig. 4B). On the other hand, a higher increase in cytosolic Ca^{2+} after NaCl shock was observed in the strain lacking Trk1 than in wild-type cells (Fig. 4B; BYT1). The deletion of both *TRK* genes even enhanced calcium accumulation in the cytosol (Fig. 4B; BYT12). A similar calcium response to NaCl stress as in the BYT12 strain was observed in the strain with five deletions (Fig. 4B; BYT12345), indicating that K^+ importers play a major role in this cellular process. Accordingly, the calculated increase in cytosolic Ca^{2+} for BYT1, BYT12 and BYT12345 were significantly higher than that obtained for wild-type cells (Fig. 4D).

The deletion of *TRK1* and to a lesser extent of *TRK2* also affected the phase of reabsorption of Ca^{2+} after reaching the NaCl-induced peak of cytosolic calcium concentration (Fig. 4B). While in the wild-type cells cytosolic Ca^{2+} decreased back to a stable level within 5 min after NaCl addition, in cells lacking Trk1 (BYT1 strain) it took more than 10 min (Fig. 4B). Even slower Ca^{2+} reabsorption occurred in cells in which both Trk transporters were absent, and independently of the presence/absence of K^+ efflux systems (Fig. 4B; strains BYT12 and BYT12345). Only about 50% of Ca^{2+} was reabsorbed in these cells within 13 min after NaCl shock (Fig. 4B).

To test whether the enhanced Ca^{2+}-cell response observed in cells without Trk transporters is not only specific to osmotic (NaCl-induced) stress, we also compared the response of wild-type and BYT12 cells to calcium stress. As shown in Fig. 5, after the addition of 0.133 M $CaCl_2$, cytosolic calcium also increased to a significantly higher level in BYT12 cells than in BY4741 cells.

All these data proved that the presence of plasma-membrane K^+ importers influences the Ca^{2+} stress response and signalling in cells much more than K^+ exporters. In contrast to the main K^+ importer Trk1, the loss of Trk2 seems to play a relatively minor role in the immediate salt-induced calcium response.

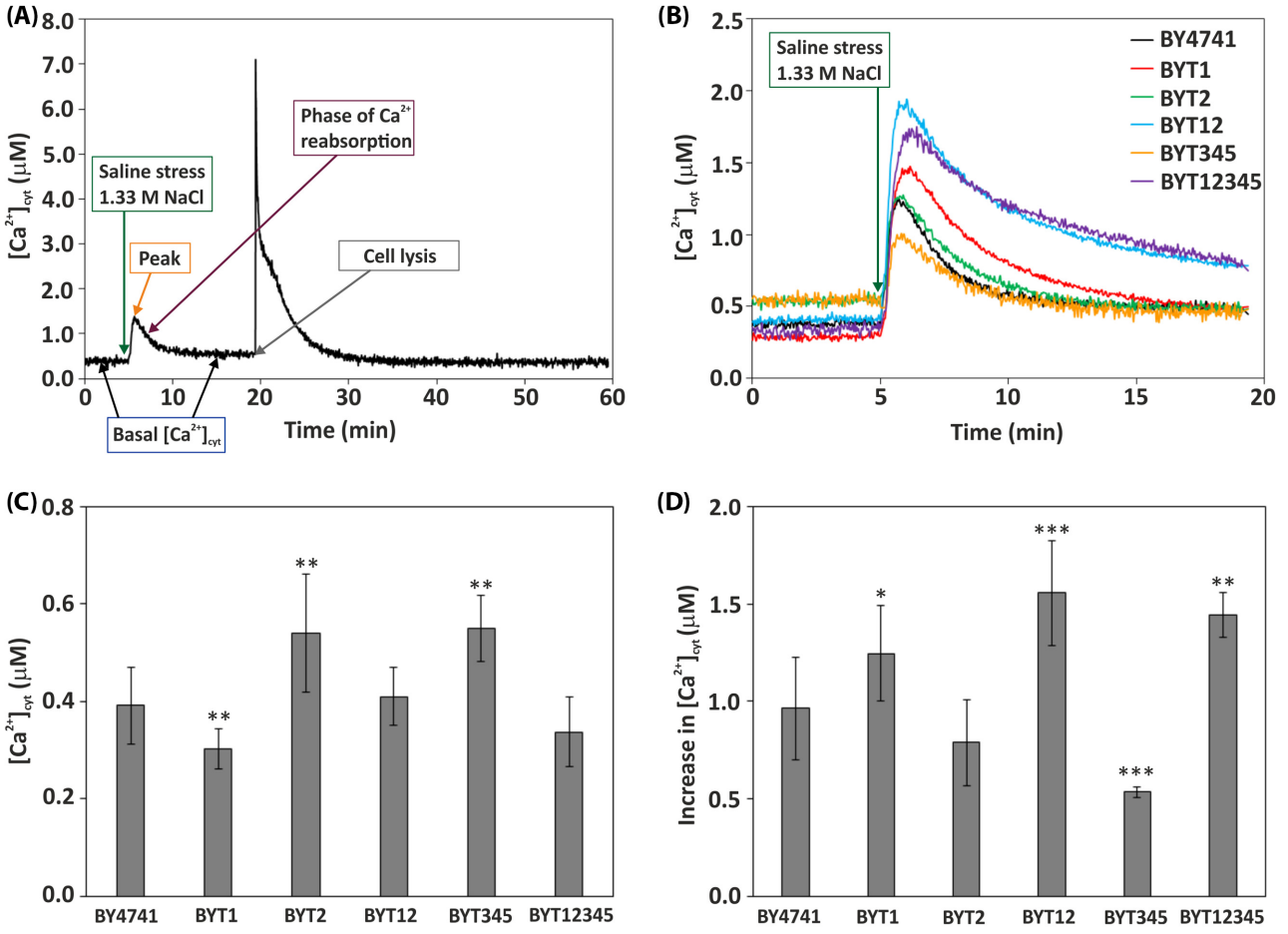

Figure 4. Ca^{2+} response to NaCl stress. Changes in $[Ca^{2+}]_{cyt}$ in the wild-type (BY4741) and strains lacking various cation transporters (BYT1 (*trk1*Δ), BYT2 (*trk2*Δ), BYT12 (*trk1*Δ *trk2*Δ), BYT345 (*tok1*Δ *nha1*Δ *ena1-5*Δ) or BYT12345 (*trk1*Δ *trk2*Δ *tok1*Δ *nha1*Δ *ena1-5*Δ)) transformed with pEVP-11-AEQ before and after the addition of NaCl (1.33 M final concentration) were monitored by aequorin-based assay. **(A)** Design of experiment with characteristic curve of changes in cytosolic Ca^{2+} in BY4741 cells over 60 min (cf. Material and Methods). **(B)** Changes in cytosolic Ca^{2+} levels in cells lacking K^+ influx and/or efflux systems. A representative curve monitoring $[Ca^{2+}]_{cyt}$ for each strain over the first 18 min of the experiment is shown. **(C)** Basal cytosolic Ca^{2+} concentration. **(D)** Increase in cytosolic Ca^{2+} concentration after NaCl shock. The values shown in (C) and (D) were calculated from 4 to 10 independent experiments for each strain. Significant differences from the increase in the wild-type strain are marked with an asterisk (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$).

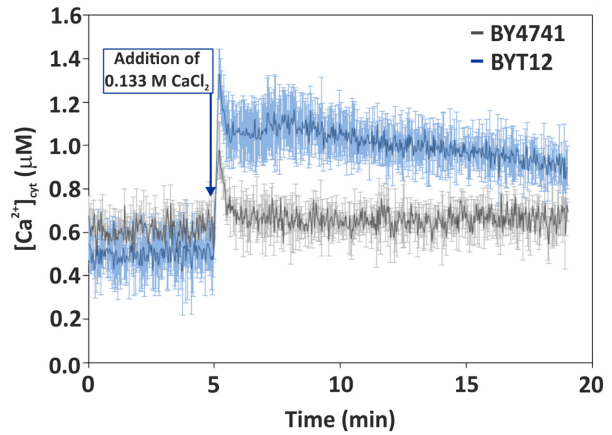

Figure 5. Ca^{2+} response to $CaCl_2$ stress. Changes in $[Ca^{2+}]_{cyt}$ in the wild-type (BY4741) and the strain lacking K^+-importers (BYT12 (*trk1*Δ *trk2*Δ)) transformed with pEVP-11-AEQ were monitored by aequorin-based assay. Shown are curves monitoring (for 18 min) changes in cytosolic Ca^{2+} before and after the addition of $CaCl_2$ (0.133 M final concentration) obtained in three independent experiments ± SD.

The absence of Trk-transporters decreases salt-stress-induced Ca^{2+} influx from extracellular environment

As was mentioned above, the transient elevation of Ca^{2+} in the cytosol triggered by high NaCl results from a cumulative transport of Ca^{2+} both from the extracellular environment via calcium transporters and/or from intracellular stocks (Denis and Cyert 2002; Matsumoto *et al.* 2002; Rigamonti *et al.* 2015). To distinguish which of these two ways is affected in cells lacking Trk transporters, we monitored changes in cytosolic Ca^{2+} during the NaCl shock in the presence of the membrane-impermeable, divalent cation chelating agent ethylene glycol tetraacetic acid (EGTA), which attenuates the transport of Ca^{2+} from the extracellular environment (Matsumoto *et al.* 2002). In the presence of EGTA, the increase in cytosolic Ca^{2+} after the addition of NaCl was largely inhibited in wild-type BY4741 cells (Fig. 6A). On the other hand, very similar courses of changes in cytosolic Ca^{2+} concentration were observed in BYT12 cells lacking both Trk1 and Trk2 transporters (Fig. 6A). The cytosolic Ca^{2+} increased to the same level in BYT12 cells after NaCl shock in the presence (Fig. 6B) and absence of EGTA (Fig. 4D). The increase in cytosolic

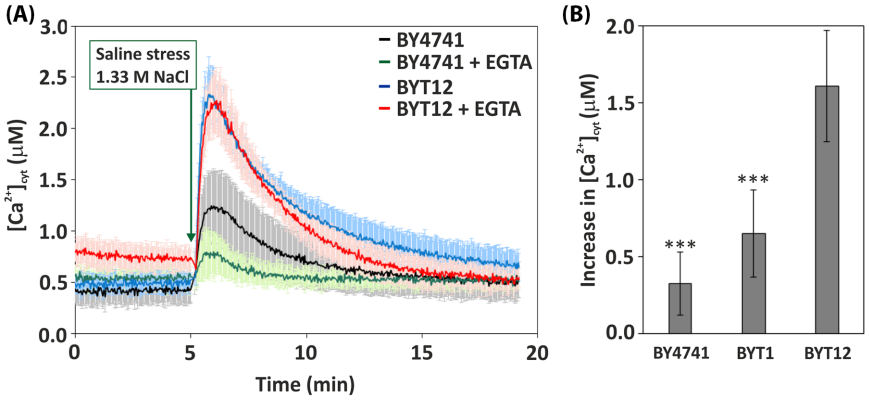

Figure 6. Effect of extracellular EGTA on Ca^{2+} response to NaCl stress. (A) Changes in $[Ca^{2+}]_{cyt}$ in the absence or presence of 10 mM EGTA (as indicated) in the wild-type (BY4741) and the strain lacking K^+ importers (BYT12 (*trk1*Δ *trk2*Δ)) transformed with pEVP-11-AEQ were monitored by aequorin-based assay. Shown are curves monitoring (for 18 min) changes in cytosolic Ca^{2+} before and after the addition of NaCl (1.33 M final concentration) obtained in at least four independent experiments ± SD. (B) Increase in cytosolic Ca^{2+} concentration after NaCl shock in the presence of 10 mM EGTA obtained in the wild-type (BY4741) and strains lacking K^+ importers (BYT1 (*trk1*Δ); BYT12 (*trk1*Δ *trk2*Δ)). The values were calculated from 5 to 7 independent experiments for each strain. Significant differences from the increase obtained for the same strain in the absence of EGTA (c.f. Fig. 4D) are marked with an asterisk (*** $P < 0.001$).

Ca^{2+} in BY4741 in the presence of EGTA (Fig. 6B) only reached 33.1% of the level obtained for wild-type cells in the absence of EGTA (Fig. 4D). When the same experiment was performed with BYT1 cells (*trk1*Δ), an intermediate phenotype between BY4741 and BYT12 was observed. The increase in cytosolic Ca^{2+} concentration in BYT1 cells in the presence of EGTA (Fig. 6B) reached 51.9% of the level obtained in BYT1 cells in the absence of EGTA (Fig. 4D). This means that the enhanced Ca^{2+}-response to NaCl stress in cells without plasma-membrane Trk potassium importers is caused by elevated Ca^{2+} transport from intracellular compartments (vacuole).

DISCUSSION

Calcium and potassium are among the most important cations ensuring various cellular functions in cells, and there is a growing list of evidence that potassium and calcium homeostasis are tightly connected in all eukaryotic cells (e.g. Liu *et al.* 2013; Rees *et al.* 2018; Liu and Martinoia 2020). Although an interconnection between K^+ transport and Ca^{2+} metabolism in yeasts has already been suggested (Mulet *et al.* 1999; Lauff and Santa-Maria 2010; Martin *et al.* 2011), this work provides a comprehensive study of the importance of individual plasma-membrane potassium transporters (importers or exporters) for Ca^{2+} homeostasis and stress-induced Ca^{2+}-signal response in *S. cerevisiae*. In general, our data show that a change in K^+ fluxes (both import and export) across the plasma membrane significantly influences Ca^{2+}-related parameters and functions. The role of particular K^+-transporters is discussed below.

We examined in detail Ca^{2+} tolerance, total Ca^{2+} content and Ca^{2+} cell response to NaCl or $CaCl_2$ stresses in a set of yeast strains with disturbed K^+ fluxes and homeostasis. Our data revealed that in particular the K^+ importers, Trk1 and Trk2 play important and distinct roles in properly setting up these cellular processes. Trk-dependent K^+-Ca^{2+} interplay is most likely not related to gene expression, as the expression of the main genes related to Ca^{2+} transport or Ca^{2+}-signalling was found to be unchanged in cells lacking both *TRK* genes (BYT12) when grown in YNB-F + 50 mM KCl (Barreto *et al.* 2012). A slight repression of *CNA1*, the gene encoding a catalytic subunit of calcineurin, was only observed in wild-type cells upon K^+-starvation (Barreto *et al.* 2012). On the other hand, the lack of *TRK1* and *TRK2* resulted in a 2-fold induction of *KCH2*, encoding one of the two small homologous plasma-membrane proteins, which contribute to the maintenance of optimal monovalent cation homeostasis and membrane potential (Felcmanova *et al.* 2017). The Kch proteins are essential for the K^+-dependent activation of the high-affinity Ca^{2+}-influx system (HACS) responding to mating pheromones (Stefan *et al.* 2013).

The lack of Trk1 and/or Trk2 resulted in high sensitivity to elevated concentrations of $CaCl_2$ (Fig. 2). In contrast to cells lacking K^+ importer(s), the strain BYT345 lacking the three plasma membrane K^+ efflux systems (Tok1, Nha1 and Ena1-5) exhibited the same phenotype as wild-type cells in terms of their tolerance to elevated external Ca^{2+} (Fig. 2). We also show that the total intracellular Ca^{2+} content was the same in wild-type and BYT345 cells (Fig. 3). A slight, but significant difference from the wild-type was observed in BYT345 in terms of the cytosolic level of Ca^{2+}, being higher in BYT345 (Fig. 4B and C). This indicates that the lack of K^+ efflux systems in the plasma membrane perhaps disturbs the balance of Ca^{2+} between the cytosol and other intracellular compartments. An elevated basal cytosolic concentration of Ca^{2+} might cause a decrease in Ca^{2+} fluxing to the cytosol (from vacuole) after NaCl shock in BYT345 compared to the wild-type strain (Fig. 4B and D). All three K^+-extrusion systems contribute to the adjustment of membrane potential, and their absence causes depolarization of the plasma membrane (Kinclova-Zimmermannova, Gaskova and Sychrova 2006; Maresova *et al.* 2006; Zahradka and Sychrova 2012). In addition, in a previous screening, *nha1*Δ cells, but not *tok1*Δ or *ena1-5*Δ cells, exhibited altered Ca^{2+} influx when grown in YPD or YPD supplemented with calcineurin inhibitor FK506, tunicamycin or α-factor (Martin *et al.* 2011). Further experiments are needed to distinguish whether the depolarization (or any other parameter changed by K^+ efflux disruption) lowers Ca^{2+} entry from external media and/or influences the transport of Ca^{2+} to/from the vacuole and/or other intracellular organelles.

Yeast Trk1 is a housekeeping protein, expressed at a low level, which provides cells with a sufficient amount of K^+ (Gaber, Styles and Fink 1988; Ko and Gaber 1991). It can work as a high or low-affinity K^+-transporter according to availability of K^+(Haro and Rodriguez-Navarro 2002). Its gene is more likely transcriptionally unregulated, but many proteins were found

to directly or indirectly regulate the Trk1 transporter at post-translational levels (Arino, Ramos and Sychrova 2019). A lack of Trk1 also hyperpolarizes the plasma membrane (Petrezselyova, Ramos and Sychrova 2011). Here, we show in detail that *trk1*Δ cells exhibit high sensitivity to external $CaCl_2$ (Fig. 2), which is in agreement with a previous systematic study, which identified a *trk1*Δ strain among 62 Ca^{2+}-sensitive mutants (Ghanegolmohammadi *et al*. 2017). We also observed that the total content of intracellular Ca^{2+} did not change with *TRK1* deletion (Fig. 3), whilst these cells contained a significantly lower cytosolic concentration of Ca^{2+} compared to the wild-type cells (Fig. 4B and C). Thus, the lack of Trk1 K^+ importer (hyperpolarizing the plasma membrane; Petrezselyova, Ramos and Sychrova 2011) seems to have the opposite effect on intracellular Ca^{2+} in the cytosol than the lack of efflux systems. This confirms that plasma-membrane potential and/or K^+ circulation/fluxes are important for the establishment of Ca^{2+} concentration in the cytosol of yeast cells. Changes in K^+ movement across the plasma membrane, and its effect on membrane potential, were also found to markedly influence cytosolic Ca^{2+} levels in endothelial cells (reviewed in Adams and Hill 2004).

The potassium uptake via Trk2 is much lower than via Trk1 in exponentially growing cells (Ramos *et al*. 1994), while it is more important for K^+ supply in the stationary phase of growth (Borovikova *et al*. 2014). As for the cell tolerance to external $CaCl_2$, Trk2 plays a less important role than Trk1. Its participation was only demonstrated on plates with very high $CaCl_2$, or when both *TRK* genes were deleted (Fig. 2; BYT12 cells). On the other hand, we show that the absence of Trk2 led to a doubled total amount of Ca^{2+} in exponentially growing cells compared to the wild type (Fig. 3). Also, the cytosolic Ca^{2+} level was higher in *trk2*Δ cells (Fig. 4B and C). Since cells lacking both *TRK* genes (BYT12 strain) alone or in combination with genes for exporters (strain BYT12345) contained a similar amount of total intracellular Ca^{2+} to the *trk2*Δ strain (Fig. 3), Trk2 plays the most important role of the five tested K^+-transporters in the determination of total intracellular Ca^{2+}. This unexpected observation indicates that Trk2 may function as sensor of Ca^{2+} similarly as was shown for other transporter-like sensors of various nutrients (Conrad *et al*. 2014). Then the absence of Trk2 somehow increases Ca^{2+} import from the extracellular environment even under the non-stressed conditions used for our total Ca^{2+} measurements (Fig. 3). As was shown previously, the continuous rise in cytosolic Ca^{2+} concentration increases the rate of vacuolar Ca^{2+} accumulation (Dunn, Gable and Beeler 1994). This would cause the observed elevation of total cellular Ca^{2+} in all three strains bearing a deletion of *TRK2* (Fig. 3). Nevertheless, future investigation is necessary to confirm this hypothesis.

Plasma-membrane hyperpolarization resulting from the deletion of either *TRK1* or both *TRK* genes implies a higher sensitivity of cells to cationic drugs and toxic monovalent cations (Navarrete *et al*. 2010; Petrezselyova, Ramos and Sychrova 2011), which was also clearly demonstrated here (Fig. 1). Nevertheless, the results obtained in the drop test on YNB-NH_4Cl-based plates (Fig. 1) revealed two new phenotypes related to the deletion of only the *TRK2* gene—increased sensitivity to a high concentration of extracellular KCl or hygromycin B (not observed previously on YPD-based plates; Petrezselyova, Ramos and Sychrova 2011). Cell sensitivity to hygromycin B is determined by membrane potential, but also by some (as-yet unknown) other parameters as was shown previously (Maresova *et al*. 2006). Sensitivity of BYT2 cells to high KCl concentration (Fig. 1) could be connected to Trk1-mediated K^+ influx activity, because it was supressed by the additional deletion of the *TRK1* gene (BYT12 cells were more tolerant to high KCl than BYT2, Fig. 1). Surplus K^+ influx via Trk1 in the presence of high extracellular KCl concentration may be toxic in *trk2*Δ cells, which already have doubled intracellular concentration of Ca^{2+} (Fig. 3). In summary, Trk2 seems to be employed in multiple physiological functions depending on the growth phase. In stationary cells, it is a major potassium supplier crucial for desiccation survival (Borovikova *et al*. 2014). On the other hand, in growing cells, in which mainly Trk1 is responsible for K^+ import (Haro and Rodriguez-Navarro 2002), Trk2 contributes relatively little to K^+ supply (Michel *et al*. 2006; Petrezselyova, Ramos and Sychrova 2011), but it is important for the maintenance of intracellular Ca^{2+} concentration and for resistance to high KCl and hygromycin B (this work).

Cellular Ca^{2+} signalling is a complex, multifactorial and dynamic process, and is critical for many physiological circumstances (Bootman and Bultynck 2020). As was mentioned in the introduction, *S. cerevisiae* cells respond to various stresses by an immediate and transient increase in the cytosolic concentration of Ca^{2+} flowing into the cytosol either from the extracellular environment or from intracellular stocks (vacuole) (Mendoza *et al*. 1994, Denis and Cyert 2002, Matsumoto *et al*. 2002, Rigamonti *et al*. 2015). However, the involvement of plasma membrane Ca^{2+} transporters in this process is somehow controversial, as Denis *et al*. reported that the addition of a Ca^{2+}-chelating compounds to the extracellular environment had no effect on the Ca^{2+} increase in the cytosol (Denis and Cyert 2002), while others (Batiza, Schulz and Masson 1996; Matsumoto *et al*. 2002; Viladevall *et al*. 2004) showed that the transient rise in cytoplasmic calcium was largely abolished by the addition of EGTA or BAPTA. In our experiments, in response to NaCl, the presence of EGTA in the extracellular medium significantly decreased the amount of Ca^{2+} accumulated in the cytosol in wild-type cells (Fig. 6), which confirms that plasma-membrane Ca^{2+} influx systems are involved in the NaCl-induced Ca^{2+}-stress response. In addition, we show that the presence of K^+ importers in the plasma membrane is crucial for the strength of the Ca^{2+}-stress response (Fig. 4). The lack of both Trk transporters not only highly increased the Ca^{2+} accumulation in the cytosol (Fig. 4B and D), but simultaneously highly diminished part of the Ca^{2+} flux through the plasma-membrane, as the response of BYT12 cells to NaCl shock was very similar in the presence or absence of EGTA (Fig. 6). Apparently, upon hyperosmotic stress, cells defective in K^+ import lack calcium influx from the extracellular environment. The high-affinity calcium transport system (HACS) exhibits structural and functional properties of a voltage-gated Ca^{2+} channel and becomes activated in response to depolarization (Espeso 2016). Thus, plasma-membrane hyperpolarization in BYT12 cells caused by the lack of Trk systems (Navarrete *et al*. 2010) may have impaired NaCl-induced Ca^{2+}-influx via HACS. The role of Trk transporters in Ca^{2+}-stress response is crucial not only upon salt stress (Fig. 4). A higher accumulation of cytosolic Ca^{2+} in the BYT12 strain than in the wild type was also observed after the addition of 1.33 M sorbitol (data not shown), 0.133 M $CaCl_2$ (Fig. 5) or upon K^+ deprivation (Lauff and Santa-Maria 2010). Thus, the presence of Trk transporters seems to be highly important for the stress induced Ca^{2+}-cell response in general. However, further experiments will be necessary to resolve whether the enhanced Ca^{2+} accumulation induced by a stress results from hyperpolarization of the plasma membrane caused by the absence of Trk, higher content of Ca^{2+} in cells (Figs 3 and 4C) and/or diminished active K^+ import to cells. In addition, Ca^{2+}-monitoring in cells lacking only one of the Trk

proteins (strains BYT1 or BYT2, respectively) showed that predominantly the lack of Trk1 enhanced the immediate cell Ca^{2+}-response to NaCl stress, while Trk2 or K^+-exporters played less important roles (Fig. 4B and D).

In conclusion, all our data proved that K^+ homeostasis and fluxes across the plasma membrane are highly important for the regulation of Ca^{2+} homeostasis and proper stress-induced Ca^{2+} signalling in *S. cerevisiae* cells. Although alterations in potassium export had a relatively small effect on the intracellular level of calcium or calcium response to hyperosmotic stress, the K^+-importers Trk1 and Trk2 play crucial and different roles in Ca^{2+} homeostasis and signalling. While the presence of Trk1 is critical for the immediate Ca^{2+}-induced cell response to stresses and cell resistance to high extracellular calcium, Trk2 transporter influences steady-state intracellular Ca^{2+} levels more. The obtained data provided new knowledge, but also raised many new questions concerning the complexity of the interplay between potassium and calcium homeostasis in yeast cells, on which we will focus in the future. Moreover, since Trk K^+ importers as well as some fungal-specific targets of the calcium signalling pathway are not conserved in humans (Arino, Ramos and Sychrova 2010; Liu *et al.* 2015; Park *et al.* 2019), our results describing crosstalk between Ca^{2+} and K^+ homeostasis may be useful for further searches for new therapies that could enhance the efficacy of our limited clinically useful antifungal drugs.

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SUPPLEMENTARY DATA

Supplementary data are available at FEMSYR online.

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Conflicts of Interest. None declared.

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