

# Trk2 transporter is a relevant player in K<sup>+</sup> supply and plasma-membrane potential control in *Saccharomyces cerevisiae*

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**Abstract** In *Saccharomyces cerevisiae*, *TRK1* and *TRK2* genes encode partially redundant K<sup>+</sup> transporters. Direct involvement in K<sup>+</sup> uptake has been shown for Trk1p since cells growing under limiting environmental K<sup>+</sup> concentrations demand its presence. The biological role of Trk2p is less understood. In our experiments, *TRK2* overexpression improved the ability of *trk1* cells to grow in low K<sup>+</sup> and led to a higher accumulation of K<sup>+</sup>. Using diS-C<sub>3</sub>(3) as a potentiometric probe, we revealed a higher hyperpolarization of *trk2* cells compared to the wild type. In addition, the deletion of *TRK2* in the *trk1* genetic background increased the cell sensitivity to hygromycin B, spermine, and TMA. Our studies reinforced the conclusion that Trk1p is the prominent K<sup>+</sup> uptake transporter and for the first time revealed that though Trk2p is much less effective, its activity contributes significantly to K<sup>+</sup> supply and the maintenance of plasma-membrane potential.

## Abbreviations

|                        |                                     |
|------------------------|-------------------------------------|
| $\Delta\psi$           | Plasma membrane potential           |
| TMA                    | Tetramethylammonium                 |
| diS-C <sub>3</sub> (3) | 3,3'-dipropylthiobarbiturate iodide |
| SD                     | Synthetic minimal medium            |

|     |                        |
|-----|------------------------|
| LB  | Luria–Bertani (medium) |
| YPD | Rich medium            |

Potassium (K<sup>+</sup>) homeostasis inside the yeast cell is crucial for many physiological functions, such as osmotic regulation, protein synthesis, and enzyme activation (Rodríguez-Navarro 2000). The plasma membrane of *Saccharomyces cerevisiae* possesses several transport systems with different substrate specificities and diverse mechanisms to maintain optimal cytosolic K<sup>+</sup> concentration ( $\approx 200$ – $300$  mmol/L).

K<sup>+</sup> uptake is mediated by the plasma membrane Trk1 and Trk2 proteins. K<sup>+</sup> accumulation in the cytosol via these systems is driven by the electrochemical H<sup>+</sup> gradient across the plasma membrane generated by H<sup>+</sup>-ATPase Pma1 (Serrano et al. 1986). Trk-like transporters are well conserved among bacteria, fungi, and plant cells. A sequence homology comparison of Trk1p and Trk2p showed  $\approx 55\%$  amino acid sequence similarity overall, sharing a much higher homology in their membrane-spanning domains and significantly differing in their length of the cytoplasmic N- and C-termini. Interestingly, Trk2p homologs prevail over Trk1 homologs in many other yeast species (for review, see Ariño et al. 2010).

Understanding of the role of Trk1p in cellular processes is relatively well developed compared to Trk2p. Phenotypic and kinetic characterizations of the Trk1 transporter provided clear evidence that Trk1p is the primary high-affinity K<sup>+</sup> transport system ( $K_m \approx 25$   $\mu\text{mol/L}$ ) (Gaber et al. 1988; Rodríguez-Navarro and Ramos 1984). The activity of Trk1p has been described to be important for K<sup>+</sup> and pH homeostases (Madrid et al. 1998; Yenush et al. 2002), turgor (Merchan et al. 2004), and plasma membrane

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potential  $\Delta\psi$  (Madrid et al. 1998; Mulet et al. 1999). Available data on the function and regulation of Trk2p are incomplete and somewhat confusing. Trk2p is not the low-affinity  $K^+$  transporter, as was proposed initially (Ko and Gaber 1991), but rather a moderate-affinity  $K^+$  transporter with a very low  $V_{max}$  (Ramos et al. 1994). The relevance of Trk2p in  $K^+$  acquisition has been questioned because of the lack of  $K^+$ -related phenotype for the single *trk2* mutant, and its exacerbated growth under  $K^+$ -limiting conditions has only been recorded in the *trk1* genetic background. The overwhelming majority of physiological works has been performed on *trk1 trk2* cells, more concerned with the study of Trk1p. The only direct evidence for the involvement of Trk2p in  $K^+$  homeostasis comes from the work of Michel et al. (2006), showing that Trk2p might play a physiological role under low  $K^+$  and pH stress in a *sin3* genetic background.

To study the function of Trk2p in more detail, we used a set of isogenic strains lacking one or both *TRK1* and *TRK2* genes in the BY4741 genetic background. Changes in  $\Delta\psi$ , cell volume, and  $K^+$  uptake, brought about by the deletion and/or overexpression of *TRK* genes, were determined under nonlimiting  $K^+$  and  $K^+$ -starvation conditions. Phenotypic studies of our strains reinforced the conclusion that Trk2p is much less active in  $K^+$  uptake than Trk1p, but its activity was clearly visible. Here, we report for the first time that Trk2p contributes to the maintenance of  $\Delta\psi$ .

## Materials and methods

**Strains and growth conditions** Used *S. cerevisiae* strains were BY4741 (*MATa his3 $\Delta$ 1 leu2 $\Delta$  met15 $\Delta$  ura3 $\Delta$* ; EURO-SCARF) and its derivatives. The *trk1* (BYT1), *trk2* (BYT2), and *trk1 trk2* (BYT12) strains were constructed using the Cre-*loxP* system (Guldener et al. 1996) as described by Petrezsélyová et al. (2010). Strains were routinely grown at 30°C either in rich (YPD) or minimal (SD) media solidified with 2% agar when necessary. YPD (1% yeast extract, 2% peptone, and 2% glucose) medium was supplemented with 50 mmol/L KCl. For  $K^+$ -dependent experiments, synthetic minimal  $K^+$ -free medium contained 0.175%  $K^+$ -free YNB (ForMedium™, UK), 2% glucose (SD) or 2% galactose (SGal), 0.4% ammonium sulfate, all necessary supplements and the indicated amounts of KCl. The pH was adjusted by the addition of ammonium hydroxide solution to 5.8. The SD medium contained  $\approx 15 \mu\text{mol/L } K^+$  and 1.3 mmol/L  $Na^+$  (J. Ramos, unpublished results). For plasmid amplification, *Escherichia coli* XL1-Blue strain was grown at 37°C in LB broth (Sigma-Aldrich) with 100  $\mu\text{g/mL}$  ampicillin.

**Plasmid constructions** The *TRK2* gene was expressed under the control of its own promoter ( $\approx 500$  bp) within the multicopy plasmid YEp352 (Hill et al. 1986). DNA fragments containing

the *TRK2* gene were amplified by polymerase chain reaction (PCR) with the oligonucleotides TRK2-YEp352-F (5'-AGC GGA TAA CAA TTT CAC ACA GGA AAC AGC TAT GAC CAT GTT CCA AGC TGG GTG GAT ACC CCG-3') and TRK2-YEp352-R (5'-CGG CCA GTG CCA AGC TTG CAT GCC TGC AGG TCG ACT CTA GTT AAT GCT TCC CCC AAA ACT TTG TTG C-3'), in which homologous regions to the YEp352 plasmid were introduced (underlined). For visualizing Trk2p, the *TRK2* gene was introduced under the control of the inducible *GAL1* promoter to the centromeric vector p416 (Mumberg et al. 1995) and tagged at its 3' terminus with a green fluorescent protein (GFP)-encoding sequence. The fragment containing *TRK2* was amplified by PCR with the oligonucleotides TRK2-pJCB-F (5'-TAC CTC TAT ACT TTA ACG TCA AGG AGA AAA AAC TAT AAT GCC AAC AGC TAA GAG GAC-3') and TRK2-pJCB-R (5'-TAC TGT TAA TTG CTC CAG CAC CAG CAC CAG CAC CTG CTC CAT GCT TCC CCC AAA ACT TTG-3'), in which homologous regions to the p416 plasmid were introduced (underlined). In both cases, the genomic DNA of *S. cerevisiae* BY4741 was used as a template. The PCR products were inserted into the YEp352 or p416 plasmids using homologous recombination.

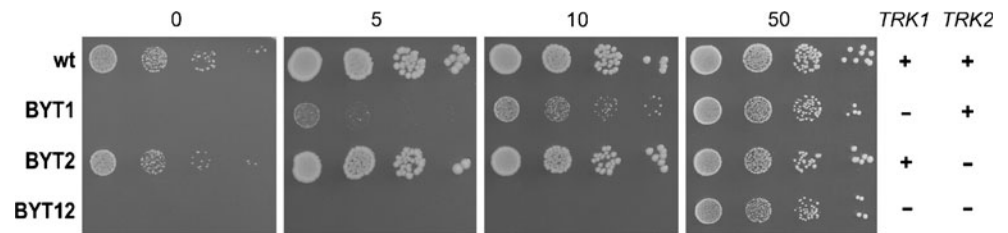
**Growth assays on solid media**  $K^+$ -dependent phenotypes were analyzed on SD media with or without the addition of KCl (1–100 mmol/L). Tolerance to toxic compounds was estimated on YPD media containing 50 mmol/L KCl, with the addition of an appropriate drug as follows: 20  $\mu\text{g/mL}$  of hygromycin B, 1.5 mmol/L spermine, or 0.4 mol/L tetramethylammonium (TMA). Serial tenfold dilutions of saturated cultures were prepared, and 3  $\mu\text{L}$  of each sample was spotted on plates. Growth was monitored for 3 days.

**Estimation of relative  $\Delta\psi$  changes** Exponential-phase cells grown in SD media were harvested, washed, and transferred into fresh SD media with or without KCl. The fluorescence assay of  $\Delta\psi$  was performed using diS-C<sub>3</sub>(3) (Marešová et al. 2009). The assay was repeated at least three times for all strains with consistent results.

**Internal [ $K^+$ ] measurements** The internal  $K^+$  content of cells starved for  $K^+$  and then grown in SD medium containing 50 mmol/L KCl was measured according to Rodríguez-Navarro and Ramos (1984). Briefly, BYT1 cells expressing the YEp352 (control) or YEp352-TRK2 plasmid were collected on Millipore filters and washed with 20 mmol/L  $MgCl_2$ . Cells were then extracted with HCl and analyzed by atomic emission spectrophotometry. The experiments were repeated three times.

**Cell size measurements** Overnight cultures from SD medium (50 mmol/L KCl) were washed and transferred into fresh SD

**Fig. 1** Growth comparison of wild-type (*wt*) and mutant strains (BYT1, BYT2, BYT12) lacking *TRK1* and/or *TRK2* genes on solid SD media containing KCl (0, 5, 10, 50 mmol/L)



medium with or without 50 mmol/L KCl. Immediately and after 60 min of incubation, cell size was determined in a Cell Counter Z2 (Beckman Coulter). The experiment was repeated at least three times, each time,  $\approx 10^6$  cells were analyzed for each strain and each condition.

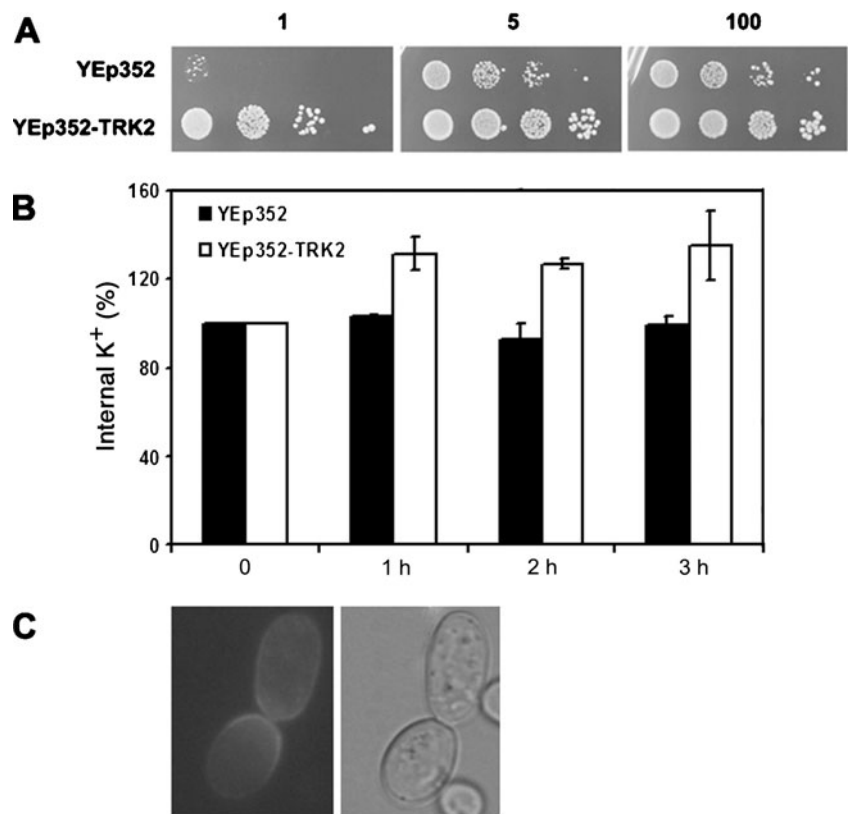
**Fluorescence microscopy** Cells exponentially growing in SGal medium (50 mmol/L KCl) were observed with an Olympus BX61 fluorescence microscope.

**Results and discussion**

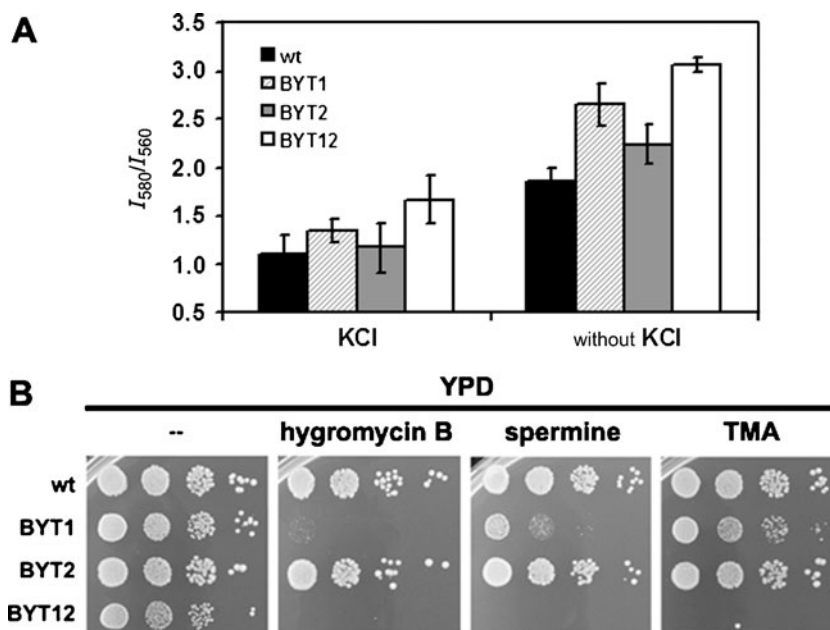
**Growth characteristics of  $K^+$  uptake-deficient mutants on  $K^+$ -defined media** Information on the function of the major  $K^+$  uptake system in *S. cerevisiae* encoded by the *TRK1* gene is abundant. On the other hand, the participation of the

product of its homolog (*TRK2*), whose  $K^+$ -homeostasis regulation is less understood, requires further investigation. In order to identify the physiological role of Trk2p, we used a set of mutant strains lacking the *TRK1* (BYT1), *TRK2* (BYT2), or both (BYT12) genes. We first examined the growth properties of single and double *trk* mutants on solid SD media containing varying amounts of  $K^+$  (Fig. 1). Accordingly to previous works, *trk1* deletion led to a pronounced growth reduction of mutant cells on SD plates containing low  $K^+$  concentrations (Ko and Gaber 1991; Bertl et al. 2003). Our BYT1 and BYT12 strains grew at similar rates to the wild type on solid SD media supplemented with 50 mmol/L KCl. Nevertheless, the growth curves of all strains from liquid SD media showed that the presence of at least 200 m mol/L KCl was required to achieve wild-type-like growth of BYT12 mutant cells (not shown). Without KCl addition (SD medium alone contains  $\approx 15 \mu\text{mol/L } K^+$ ), only cells with a functional Trk1

**Fig. 2** Effect of *TRK2* over-expression in BYT1 (*trk1*) cells. **a** Growth characteristics under  $K^+$ -limiting and suboptimal concentrations (1, 5, and 100 mmol/L). **b** Internal  $K^+$  content (percent) measured over time-course of 3 hours in SD medium upon  $K^+$  starvation. **c** Localization of the overexpressed GFP-tagged Trk2p in BYT2 cells



**Fig. 3** Comparison of wild-type and mutant cells lacking *TRK1* and/or *TRK2* genes in  $\Delta\psi$ -related phenotypes. **a** Estimation of relative  $\Delta\psi$  ( $I_{580}/I_{560}$ ; fluorescence emission ratio of diS-C3 (3)) of cells incubated for 3 h in SD medium with or without 50 mmol/L KCl. **b** Growth characteristics on solid YPD media containing 50 mmol/L KCl and cationic drugs



system (wild-type and BYT2 strains) were able to cope with scarce sources of  $K^+$ . Our results also clearly show that Trk2p contributes to  $K^+$  uptake since (1) the presence of the *TRK2* gene in the *trk1* strain (BYT1) facilitated the growth on medium containing 5 mmol/L KCl, and (2) the deletion of both the *TRK1* and *TRK2* genes led to a significant growth arrest in contrast to BYT1 strain when propagated on SD plates containing 5 or 10 mmol/L KCl (Fig. 1).

*Overexpression of TRK2 compensates for the  $K^+$  uptake deficiency of cells lacking TRK1* The ability of Trk2p to reduce the intracellular  $K^+$  deficit of a strain lacking the main  $K^+$  uptake system was examined by overexpression of *TRK2* gene. As shown in Fig. 2a, plating the BYT1 strain overexpressing *TRK2* from its own promoter in a multicopy plasmid on SD media containing low KCl concentrations resulted in significant suppression of the  $K^+$ -dependent growth defect of BYT1 cells. This phenomenon correlates with an increase in internal  $K^+$  content of cells starved for  $K^+$  (at least for 2 h) prior to cultivating under nonlimiting  $K^+$  concentrations (50 mmol/L KCl) (Fig. 2b). Our results show that Trk2p modulates intracellular  $K^+$  levels and contributes to  $K^+$  supply. Similar results obtained with cells lacking Sin3p, a transcriptional repressor of Trk2p, in the FY833 genetic background led to conclusion that the *TRK2* gene is normally repressed by Sin3p (Michel et al. 2006). Here, we show that, in the BY4741 genetic background, *TRK2* is expressed in *SIN3* cells growing in SD medium, and the level of observed activity depends on its gene copy number and consecutively on an increased amount of Trk2p in the plasma membrane (Fig. 2c). Whereas GFP-tagging of

the chromosomal *TRK2* copy is not sufficient to visualize the protein (Huh et al. 2003), overexpression of the GFP-tagged version results in a strong plasma membrane signal (Fig. 2c). Altogether, our *TRK2* overexpression studies brought about clear phenotypes of increased  $K^+$  uptake activity and an enhanced amount of Trk2p in the plasma membrane. Thus, the apparent low activity of Trk2p in the BY4741 strain under our conditions is due to a very low amount of the protein in the plasma membrane.

*Trk2p contributes to the maintenance of  $\Delta\psi$   $K^+$  uptake* is believed to be a major consumer of electrical potential across the plasma membrane that is generated by Pma1  $H^+$ -ATPase. However, previous studies have only provided direct evidence for the involvement of *TRK1* in  $\Delta\psi$  (Madrid et al. 1998). In order to clarify whether the activity of Trk2p also contributes to  $\Delta\psi$ , we monitored the relative  $\Delta\psi$  values of the wild-type and all mutant cells in the presence or absence of KCl using a diS-C<sub>3</sub>(3) assay. As expected, the

**Table 1** Cell volume (fL) of wild-type and mutant strains lacking *TRK1* or *TRK2* in SD media with 50 mmol/L KCl or without  $K^+$  (immediately and after a 60-min incubation)

| Strain                    | 50 mmol/L KCl | $K^+$ -free |            |
|---------------------------|---------------|-------------|------------|
|                           | 0 min         | 0 min       | 60 min     |
| wt ( <i>TRK1 TRK2</i> )   | 45.31±0.47    | 43.03±0.13  | 44.01±0.15 |
| BYT1 ( <i>trk1 TRK2</i> ) | 46.92±0.81    | 46.56±0.13  | 46.20±0.16 |
| BYT2 ( <i>TRK1 trk2</i> ) | 45.06±0.14    | 40.68±0.15  | 44.07±0.73 |

Cell volumes are means ± SD of three independent experiments

BYT12 strain displayed the most hyperpolarized state under both incubation conditions (Fig. 3a). Surprisingly, the observed hyperpolarization of BYT12 cells was evidently caused by the deletion of both, *TRK1* and *TRK2* genes, since the corresponding single deletion mutants were more hyperpolarized than the wild-type cells (this effect was more obvious in  $K^+$ -starved cells). Our data seem to contradict the recent work of Peña et al. (2009) who have reported that the *trk1* mutant did not differ from the wild type in its uptake of diS-C<sub>3</sub>(3), and the hyperpolarization of the mutant was only observed upon the addition of amiodarone, a drug which elicits dose-dependent  $K^+$  efflux (Peña et al. 2009), and the plasma membrane hyperpolarization (Marešová et al. 2009). We believe that the contradiction is caused by differing growth conditions. Peña et al. (2009) cultivated strains in rich (YPD) medium. The lack of measurable hyperpolarization of the *trk1 trk2* mutant grown in YPD was also observed in our lab, and at least 30 min of  $K^+$  starvation was required to detect any hyperpolarization of these cells compared to the wild type (not shown). In our experiments, the use of special synthetic minimal medium made it possible to observe small but highly reproducible differences in relative values of  $\Delta\psi$  in the strains, and these differences increased upon  $K^+$  starvation (Fig. 3a).

The *trk1 trk2* mutant has been previously shown to be sensitive to hygromycin B, spermine, and TMA (e.g., Madrid et al. 1998; Yenush et al. 2002). All these molecules can be accumulated in cells in amounts dependent on the value of  $\Delta\psi$  because of their positive charge. Therefore, our collection of *trk* mutants and the wild type were also tested for their sensitivity to the above compounds. Drop tests were performed on YPD plates that contained sufficient  $K^+$  for BYT12 growth. Indeed, a difference in sensitivity to all cationic drugs among our mutants was observed, and the results showed that *trk2* deletion increased the sensitivity of *trk1* cells (Fig. 3b). Taken together, we showed that other transport systems, in addition to Trk1p, participate in  $\Delta\psi$  control and Trk2p is a relevant player in this process.

*Trk2p does not play a role in cell volume control* It has been previously proposed that mutant cells lacking Ppz1p and Ppz2p (negative regulators of *TRK1* and *TRK2* genes) are larger due to increased intracellular  $K^+$  levels (and thus due to increased turgor pressure; Merchan et al. 2004). Normal  $K^+$  content and cell size have been restored by the additional deletion of *TRK1* and *TRK2* genes in *ppz1 ppz2* cells. We asked ourselves whether Trk1p or Trk2p alone or both of them are important in cell volume control. The following experiment was carried out to verify this. Wild-type and single-deletion mutant cells were grown overnight in SD medium (50 mmol/L KCl), harvested, washed, and resuspended in fresh SD medium with or without KCl. Cell volume was measured with samples withdrawn immediately

and after 60 min of incubation in the fresh media. Under standard growth conditions in the presence of 50 mmol/L KCl, cells of the wild-type and BYT2 (*trk2*) strains were  $\approx 45$  fL large, while BYT1 (*trk1*) cells were a bit larger (Table 1). Transfer to  $K^+$ -free media caused a significant shrinkage (decrease in cell volume of  $\approx 5$ –10%) of cells expressing Trk1p (wild-type and BYT2 strains) in the beginning of  $K^+$  starvation. This rapid cell shrinkage was probably due to the loss of cell water and was reversible after another 60 min during which the majority of the cells ( $\approx 75$ –85%) came back to their original volume. In contrast, cells lacking Trk1p and expressing only the Trk2 transporter (BYT1) did not change in size upon transfer to  $K^+$ -free medium, i.e., Trk2p is probably not required for the response of cells to an altered osmotic condition.

In conclusion, our results demonstrated that the Trk2 plasma membrane protein, in spite of its limited level in cells, is involved in  $K^+$  requisition and  $\Delta\psi$  regulation in BY4741 cells grown under standard conditions, and that it is not, in contrast to Trk1p, involved in cell shrinkage upon osmotic downshift.

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