Construction of a yeast-based rescue system for screening of effects of pharmacological agents on TWIK channels

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Construction of a Yeast-Based Rescue System for Screening of Effects of Pharmacological Agents on TWIK Channels

by

Mosi Lin

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Abstract

Two-pore domain potassium (K$_{2p}$) channels conduct leak or background K$^+$ currents, which primarily maintain resting membrane potential and regulate cellular excitability. Among the TWIK subfamily of K$_{2p}$ channels, TWIK-1 (K$_{2p1}$) and TWIK-2 (K$_{2p6}$) channels are characterized as weak inwardly rectifying K$^+$ channels, whereas TWIK-3 (K$_{2p7}$) channels do not produce detectable currents in heterologous expression systems. Although K$_{2p}$ channels were first identified in the 1990s, only a few K$_{2p}$-specific pharmacological agents are available. In this study, I developed a yeast-based rescue system to screen the effects of pharmacological agents on TWIK channels. First, I employed K$^+$-uptake-deficient SGY1528 yeasts that K$^+$ transporters Trk1 and Trk2 are genetically deleted. SGY1528 yeast cannot grow in agar plates or culture medium with low extracellular K$^+$ concentrations ([K$^+$]$_o$). Heterologous expression of TWIK-1, TWIK-2, or TWIK-3 channels rescued growth of SGY1528 yeast, supporting that TWIK channels produce K$^+$ uptake in SGY1528 yeasts. Second, we studied the effects of pharmacological agents on TWIK channels expressed in SGY1528 yeasts by monitoring growth curves of these yeasts in the absence and presence of pharmacological agents. As Triton X-100 has been previously reported to inhibit some K$^+$ channels with either inward or outward currents, it is examined here for its potential inhibition on TWIK channels. Application of 0.5% Triton X-100 caused a ring-shaped inhibition zone of SGY1528 yeasts expressing TWIK-1, TWIK-2, or TWIK-3 channels on agar plates with low [K$^+$]$_o$ in a dose-dependent manner. Application of Triton X-100 also inhibited growth of TWIK-expressing SGY1528 yeast in culture medium with low [K$^+$]$_o$. In contrast, application of Ba$^{2+}$ or quinine, which are not potent inhibitors of TWIK channels, had no effect on growths of TWIK-expressing SGY1528 yeasts. Therefore, I constructed a yeast-based system that can be potentially used for high-throughput screening effects of chemical compounds on TWIK channels.
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1 INTRODUCTION

1.1. Two-pore domain Potassium Channels (K\textsubscript{2P})

Potassium (K\textsuperscript{+}) channels are the most widely distributed ion channels and play an indispensable role in regulating cellular excitability (Alberts \textit{et al.}, 2002). A subfamily of K\textsuperscript{+} channels, the two-pore domain K\textsuperscript{+} channels (K\textsubscript{2P}) that are encoded by \textit{KCNK} genes, take part in the maintenance of resting membrane potential (Thiriet, 2012). The K\textsubscript{2P} family contains 15 different members found in the human genome (Figure 1). They are categorized into six subfamilies according to their amino-acid sequences and functions: TWIK (tandem of pore domains in a weak inwardly rectifying K\textsuperscript{+} channel), TREC (TWIK-related K\textsuperscript{+} channel) and TRAAK (TWIK-related arachidonic acid-activated K\textsuperscript{+} channel), TALK (TWIK-related alkaline pH-activated K\textsuperscript{+} channel), TASK (TWIK-related acid-sensitive K\textsuperscript{+} channel), THIK (TWIK-related halothane-inhibited K\textsuperscript{+} channel), and TRESK (TWIK-related spinal cord K\textsuperscript{+} channel) (Enyedi & Czirjak, 2010).

Since the first ion channel was cloned in 1984, ion channels have received much attention as a target for drug discovery and development (Owen & Silverthorne, 2002). K\textsubscript{2P} channels are known as background K\textsuperscript{+} channels that stabilize resting membrane potential close to K\textsuperscript{+} equilibrium potential (Gonzalez \textit{et al.}, 2012); thus, they provide valuable targets for potential therapies (Bayliss & Barrett, 2008). As one class of K\textsubscript{2P} channels, TWIK channels also play fundamental roles in physiology and are a potential target of pharmacological agents.
Figure 1. Phylogenetic tree of mammalian K$_{2P}$ channels with six subfamilies and 15 members.

TWIK channels (TWIK-1, TWIK-2, and TWIK-3) are composed of K$_{2P}$1.1, K$_{2P}$6.1, and K$_{2P}$7.1, named by the International Union of Pharmacology (IUPHAR). They are encoded by KCNK1, KCNK6, and KCNK7 genes accordingly, by the HUGO Gene Nomenclature Committee (HGNC) (Goldstein et al., 2005; Kindler & Yost, 2005)). Adopted and modified from (Enyedi & Czirjak, 2010).
1.2. TWIK Channels

TWIK-1 channels were the first identified mammalian K$_{2P}$ channels (Lesage et al., 1996a), following the discovery of K$_{2P}$ channels in non-mammal organisms, which are TOK1 from *Saccharomyces cerevisiae* (Ketchum et al., 1995) and KCNK0 from *Drosophila* (Goldstein et al., 1996). TWIK-2 and TWIK-3 channels were discovered in 1999 (Chavez et al., 1999; Pountney et al., 1999; Salinas et al., 1999). Like other K$_{2P}$ channels, TWIK channels contain two pore-domains (P1 and P2) and four transmembrane segments and they do not have voltage sensors (Sepulveda et al., 2015). All of them have the K$^+$ channel signature sequence TxGYG in the P1 region (Figure 2). TWIK-1, TWIK-2, and mouse/rat TWIK-3 have the GL(Y)G motif in the P2 region (Lesage et al., 1996b; Salinas et al., 1999). Human TWIK-3 contains an unconventional GLE sequence in the P2 region (Salinas et al., 1999), which may be related to its altered ion selectivity and resulted in its inactivity (Chen et al., 2014a).
Figure 2. Alignment of two pore-domains of human, mouse, and rat TWIK channels.

Amino acid sequences of the P1 and P2 regions of indicated TWIK-1, TWIK-2, and TWIK-3 channels are aligned. Pore helices are indicated in blue and selectivity filters are in orange. Adopted and modified from (Salinas et al., 1999; Gonzalez et al., 2012; Chen et al., 2014a).
TWIK channels are leak channels that open at all potentials (Goldstein et al., 2001; Niemeyer et al., 2016). TWIK-1 channels mediate an instantaneous and non-inactivating current (Lesage et al., 1996a; Lesage et al., 1997), while TWIK-2 channels are inactivated at depolarized potentials (Patel et al., 2000). Under physiological [K+]o conditions, TWIK-1 and TWIK-2 channels exhibit linear current-voltage relationships at both positive and negative voltages (Lesage et al., 1996a; Lesage et al., 1997; Rajan et al., 2005; Lloyd et al., 2009). They conduct inward or outward currents upon hyperpolarization or depolarization, respectively (Lesage et al., 1996a; Patel et al., 2000). TWIK-3 channels do not generate functional currents when expressed in mammalian heterologous systems (Salinas et al., 1999; Bobak et al., 2017). The wild-type TWIK-1 only induced small, weak rectifier currents when it was first expressed in Xenopus oocyte (Lesage et al., 1996a). However, studies have shown that the small currents are due to the low levels of expression of TWIK-1 in plasma membrane and sumoylation of the residue Lys274. TWIK-1-K274E mutation resulted in a robust current (Decressac et al., 2004; Rajan et al., 2005; Feliciangeli et al., 2010; Bobak et al., 2017). Another explanation for small currents is a hydrophobic barrier in the inner pore of TWIK-1 channels, in which a mutation of the residue Leu146 increases the hydration of the channel and the currents sequentially (Aryal et al., 2014). The hydrophobic barrier is also related to the mechanism of current inhibition with quinine and quinidine (Wulff & Zhorov, 2008).
Figure 3. Effects of Ba\(^{2+}\) and quinine on rat TWIK-1-K274E and TWIK-2 channels.

(A) Whole-cell current recording of rat TWIK-1-K274E channels expressed in CHO cells with 800 µM Ba\(^{2+}\) (grey line) or with 1 mM quinine (purple line), and without anything (black line) under physiological [K\(^{+}\)]\(_{o}\) condition. 800 µM Ba\(^{2+}\) inhibits 50% of TWIK-1-K274E K\(^{+}\) currents with an IC\(_{50}\) of 960 µM, while 1 mM quinine inhibits the currents of TWIK-1-K274E with IC\(_{50}\) values of 85 µM. (B) Whole-cell current recording of rat TWIK-2 channels expressed in CHO cells with 100 µM Ba\(^{2+}\) (grey line) or with 1 mM quinine (purple line), and without anything (black line) under physiological [K\(^{+}\)]\(_{o}\) condition. TWIK-2 K\(^{+}\) currents show higher sensitivity to extracellular Ba\(^{2+}\) in physiological [K\(^{+}\)]\(_{o}\) condition with an IC\(_{50}\) of 100 µM, while quinine inhibits the currents of TWIK-2 with IC\(_{50}\) values of 100 µM. Adopted and modified from (Lloyd et al., 2009; Zhou et al., 2009; Chen et al., 2014b).
TWIK channels are expressed in various tissues. TWIK-1 is abundant, particularly in the brain, heart, and lung (Arrighi et al., 1998; Wang et al., 1998; Lesage & Lazdunski, 2000; Medhurst et al., 2001). TWIK-3 shows a similar distribution as TWIK-1, preferentially in brain and lung, but with an overall low-level expression (Lesage & Lazdunski, 2000; Medhurst et al., 2001). TWIK-2 is mainly expressed in the pancreas, spleen, stomach, and prostate, but weakly expressed in the brain stem and central nervous system (Lesage & Lazdunski, 2000; Patel et al., 2000; Medhurst et al., 2001).

Several studies have proven that TWIK-1 and TWIK-2 channels regulate numerous physiological processes and are associated with different diseases. TWIK-1 channels play a role in endocytosis (Decressac et al., 2004) and regulation of phosphate and Ca\(^{2+}\) transport (Nie et al., 2005; Yeon et al., 2015). They are also involved in hypokalemia (Ma et al., 2011a; Chatelain et al., 2012; Ma et al., 2012), nervous system diseases (Pollema-Mays et al., 2013; Mao et al., 2017), cardiac diseases (Iwasa et al., 2001; Es-Salah-Lamoureux et al., 2010) and cancer (Beitzinger et al., 2008; Williams et al., 2013). In addition, abnormal expression of TWIK-2 is associated with pulmonary hypertension (Pandit et al., 2014), vascular dysfunction (Lloyd et al., 2011), hearing loss (Mhatre et al., 2004) and cancer (Williams et al., 2013).
1.3. Pharmacology of TWIK Channels

Only a few non-specific blockers have been identified to inhibit TWIK channels (Lotshaw, 2007). TWIK-1 channels are sensitive to local anesthesia bupivacaine (O'Connell et al., 2002), while TWIK-2 channels are sensitive to inhalational anesthetics. Besides, TWIK-2 inward K⁺ currents are blocked by Cs⁺ (Patel et al., 2000). Both TWIK-1 and TWIK-2 channels are inhibited by extracellular acidification, barium ions (Ba²⁺), quinine and quinidine (Lesage et al., 1996a; Patel et al., 2000; Rajan et al., 2005).

1.3.1. Blockers for TWIK channels

1.3.1.1. Quinine

TWIK channels are sensitive to quinine at micromolar concentrations. Quinine blocks TWIK K⁺ currents by binding in their inner pores as an open-state blocker, which may be associated with the hydrophobic barrier within the inner pore (Snyders & Yeola, 1995; Wulff & Zhorov, 2008; Schmidt et al., 2013; Aryal et al., 2014). Human TWIK-1 K⁺ currents are inhibited by quinine with a half-maximal inhibitory concentration (IC₅₀) of 50 μM (Lesage et al., 1996a), and 35% of mouse TWIK-1 K⁺ currents are inhibited by 100 μM quinine (Lesage et al., 1997). Desumoylated TWIK-1 has a similar inhibitory effect by quinine. Rat TWIK-1-K274E K⁺ currents are inhibited with an IC₅₀ of 85 μM (Figure 3A) (Zhou et al., 2009). Rat TWIK-2 K⁺ currents, but not human TWIK-2 currents, are also inhibited by quinine with an IC₅₀ of 100 μM (Figure 3B) (Chavez et al., 1999; Patel et al., 2000; Chen et al., 2014b).

1.3.1.2. Barium ion

Ba²⁺ blocks TWIK-1 channels by binding inside or outside of the selectivity filter (Alagem et al., 2001; Ma et al., 2011a; Ma et al., 2011b). Therefore, sumoylation observed near the selectivity filter of TWIK-1 channels has a great effect on Ba²⁺ inhibition. Human TWIK-1 K⁺ currents are
inhibited by Ba$^{2+}$ with a low IC$_{50}$ of 100 μM (Lesage et al., 1996a) and mouse TWIK-1 K$^+$ currents are also inhibited by Ba$^{2+}$ with a low IC$_{50}$ of 35.2 μM (Lesage et al., 1997). In addition, Ba$^{2+}$ also inhibits rat TWIK-2 channels with an IC$_{50}$ of 100 μM (Chavez et al., 1999; Patel et al., 2000).

1.3.2. Triton X-100

Triton X-100 (TX-100; C$_{14}$H$_{22}$O(C$_2$H$_4$O)$_n$, n=9-10) is an amphiphilic detergent possessing a hydrophobic carbohydrate chain and a hydrophilic polyethylene glycol chain (Hollerer-Beitz & Heinemann, 1998). Previous reports have shown that detergents induce an inhibitory effect on ion channels (Depenbusch et al., 1983; Natochin et al., 1990; Narang et al., 2013). In addition, amphiphilic detergents have been observed to affect K$^+$ channels (Hollerer-Beitz & Heinemann, 1998; Narang et al., 2013). As an amphiphile, TX-100 takes various actions on K$^+$ channels depending on its concentration. When the concentration of TX-100 above its critical micellar concentration (0.01% (w/v)), it has the ability to lyse or solubilize membranes of living cells to extract proteins or organelles (Hjelmeland & Chrambach, 1984; Harshman et al., 1989). With the addition of concentration, it causes toxicity to the cells (Koley & Bard, 2010). However, at a low concentration below its critical micellar concentration, TX-100 inhibits voltage-gated K$^+$ channels (Kv) Kv2.1 or Kv1.5 with a reduction of the peak currents and a current inactivation. The current reduction was also observed when applied TX-100 at a low concentration on inward-rectifying channel Kir2.1 and outward-rectifying channel EAG. TX-100 blocks those K$^+$ channels as an open-channel blocker, which functions similarly to local anesthetics and antiarrhythmic agents (Hollerer-Beitz & Heinemann, 1998). Regarding that TWIK channels are targets for antiarrhythmic drug quinidine and quinine, and local anesthetics bupivacaine, it is, therefore, proposed that TWIK channels may be sensitive to TX-100.
1.4. Potassium-Uptake-deficient Yeast Rescue System

To search for new drugs targeting ion channels, there are several technologies that could be used: electrophysiological assays with patch-clamping techniques, fluorescence and radiotracer-based assays, and cell viability assays (Denyer et al., 1998). Additionally, various high-throughput screening approaches were developed in the last couple of decades (Zheng et al., 2004; Terstappen et al., 2010). Among these methods, electrophysiological assays, indeed, provide more precise measurements and results; however, even automated electrophysiological systems are limited by their relatively low-throughput capacity (Denyer et al., 1998; Xu et al., 2001; Bell & Dallas, 2018). To efficiently identify ion channel modulators from a chemical library, the phenotypic screening methods, like cell viability, provide the potentials as rapid alternative screening approaches (Denyer et al., 1998; Handler et al., 2018). In the past two decades, a K⁺-uptake-deficient yeast strain has been employed as a cellular model to study the pharmacology of background K⁺ channels such as Kir2.1 channels and TREK-1, another K₂P channel (K₂P2.1) (Bagriantsev et al., 2013).

1.4.1. K⁺ Transport in S. Cerevisiae

To serve as an experimental system, yeast has several advantages among all the eukaryotic cells. The genome sequence of yeasts has been fully studied, and its ionic transport system was uncomplicated and well-conserved from yeast to higher organisms, such as fungi and plants (Arino et al., 2010). In S. cerevisiae, two K⁺ transporters are mainly responsible for K⁺ influx, Trk1 and Trk2 (Figure 4) (Gaber et al., 1988; Ko et al., 1990). Trk1 and Trk2 transporters allow K⁺ transport across the membrane when the range of [K⁺]₀ is from 10 µM to 2.5 M (Yenush, 2016). To hold the steady-state intracellular K⁺ concentration ([K⁺]ᵢ), the K⁺ influx system coordinates with H⁺-ATPase (Pma1) and K⁺ efflux system (Tok1, Ena1, and Nha1) to maintain the balance of K⁺ and
protons (Figure 4), and to set membrane potential close to -100 mV – -200 mV, which value has not been confirmed experimentally but was estimated from recordings of [K\(^+\)], from other organisms, *Neurospora crassa* and plants (Rodríguez-Navarro, 2000; Volkov, 2015).

1.4.2. Expressing K\(^+\) Channel in K\(^+\)-uptake-deficient Yeast

When two major K\(^+\) transporters, Trk1 and Trk2, are knocked out, the yeast strains (*trk1Δtrk2Δ*) become deficient in K\(^+\) uptake (Anderson *et al.*, 1992; Ko *et al.*, 1993; Bihler *et al.*, 2002; Yadav *et al.*, 2015). Some nonselective channels are capable of mediating K\(^+\) influx, like Pmp3, NSC1, Qdr2, Kch1 and Kch2 (Figure 4), only in high [K\(^+\)]\(_o\) (Figure 5) (Arino *et al.*, 2010; Yenush, 2016). Because *trk1Δtrk2Δ* mutant strains only survive under high [K\(^+\)]\(_o\) conditions (50 – 100 mM), not under low [K\(^+\)]\(_o\) conditions (Figure 5), researchers heterologously expressed background K\(^+\) channels to restore the ability of K\(^+\) uptake and rescue growth of the mutant yeast under low [K\(^+\)]\(_o\) conditions (Figure 6).

One K\(^+\)-uptake-deficient yeast strain, SGY1528, was built by inserting non-functional alleles into the *TRK1* and *TRK2* locus (Tang *et al.*, 1995). It was typically used as a precursory experimental tool for screening modulators for conventional background K\(^+\) channels, the Kir2.1 channels that mediate rectifying K\(^+\) inward currents (Zaks-Makhina *et al.*, 2004; Bagriantsev *et al.*, 2013). Recent studies revealed that heterologous expression of TREK-1 channels was capable of rescuing the growth of SGY1528 in low [K\(^+\)]\(_o\) conditions (0.5 – 2 mM) by directly measuring living yeasts with a plate reader. Screening of pharmacological effects of a chemical library on growth of TREK-1-expressing SGY1528 yeasts identified several TREK-1 inhibitors (Bagriantsev *et al.*, 2011; Bagriantsev *et al.*, 2013). Like TREK-1 channels, TWIK channels also exhibits “leak” nature that mediates inward and outward currents, so it can be proposed that TWIK-expressing SGY1528 yeasts have the ability to grow under low [K\(^+\)]\(_o\) conditions and can be used as a tool for
screening of pharmacological effects of reagents on TWIK channels.

In the present study, I heterologously express TWIK channels in K⁺-uptake-deficient SGY1528 yeasts, to rescue the growth of these yeasts in low [K⁺]₀ conditions. I also test the strategy to use such SGY1528-based rescue systems to screen the effects of pharmacological agents on TWIK channels by monitoring growth curves of the TWIK-expressing SGY1528 yeasts in low [K⁺]₀ conditions in both solid and liquid media.
Figure 4. Schematic representation of $K^+$ transport system and other ionic transport systems in *S. cerevisiae*.

The major plasma-membrane and intracellular transporters for $K^+$ (purple letters), proton (grey letters), and $Na^+$ (grey letters) are indicated. In *S. cerevisiae*, Trk1 and Trk2 $K^+$ transporters are two major components for $K^+$ influx with high-affinity, while Pmp3 (plasma membrane proteolipid 3), Qdr2 (quinidine resistance protein 2), Kch1 and Kch2 (Cch1 $K^+$ regulator), and NSC1 (nonspecific cation channel) are low-affinity transporters for $K^+$ uptake. The $K^+$ efflux system is composed of Tok1, Ena1, and Nha1. *Adopted and modified from* (Arino et al., 2010; Yenush, 2016; Locascio et al., 2019).
Figure 5. Potassium concentrations required for growth of K\(^+\)-transporter mutant strains.

Different yeast strains were incubated in the mediums with indicated concentrations at 30°C for 2 days. Wild-type (TRK1 TRK2) and trk2 mutant (TRK2 trk2Δ) grew in all [K\(^+\)]\(_{o}\) conditions, and trk1 mutant (TRK1 trk1Δ) grew in all conditions except the extremely low [K\(^+\)]\(_{o}\) condition (0.2 mM KCl). While trk1Δtrk2Δ double-mutant strain only survived in the high [K\(^+\)]\(_{o}\) condition with 100 mM KCl. Adopted and modified from (Ko & Gaber, 1991).
Figure 6. Rescued growths of *trk1Δtrk2Δ* yeasts expressing Kir2.1 channels.

*K*+-uptake-deficient strains expressing different K*+* channels grew on low [K*+]o condition (0.5 mM KCl with or without 10 mM BaCl2) at 30°C for 2–3 days. Functional K*+* channels, Trk1, Kir2.1 and Kir2.1 mutants (Ba #1, Ba #2, and Ba #3), rescued the growth under low [K*+]o conditions, whereas nonfunctional K*+* channels, Kir2.1 P-stuffer, failed to rescue the growth. In the presence of Ba2+, the rescued growths of Ba2+-sensitive K*+* channels were prevented under low [K*+]o condition. The yeast expressing Trk1 or Ba2+-resistant mutant survived the Ba2+ inhibition. Adopted from (Chatelain et al., 2005).
2 MATERIALS AND METHODS

2.1. Chemicals and Reagents

Adenine Heminisulfate (Sigma-Aldrich)

Agar (BD)

Agarose LE (American Bioanalytical)

Ampicillin (Sigma-Aldrich)

L-Arginine (Free Base) (Amresco)

BaCl$_2$ (Sigma-Aldrich)

Biotin (Sigma-Aldrich)

Boric Acid (JT Baker)

CaCl$_2$ (Fisher)

CSM Dropout Uracil and Methionine Powder (Sunrise Science)

CuSO$_4$ (Sigma)

DMSO (Sigma-Aldrich)

1kb DNA Ladder (New England Biolabs)
Ethidium Bromide (Sigma-Aldrich)
FeCl₃ (Sigma-Aldrich)
QIAquick Gel Extraction Kit (Qiagen)
Gel Loading Dye (New England Biolabs)
D-Glucose (Amresco)
L-Glutamic Acid (Sigma-Aldrich)
Glycerol (Sigma-Aldrich)
HCl (JT Baker)
KCl (JT Baker)
KI (Aldrich)
Inositol (Sunrise Science)
Isopropanol (Sigma-Aldrich)
LiCl (USB)
MgCl₂ (Sigma)
MgSO₄ (Fisher)
MnSO₄ (Sigma)
Molybdic Acid (Sigma-Aldrich)

NaCl (JT Baker)

NaOH (JT Baker)

NEBuffer 2.1 (New England Biolabs)

D-Pantothenic Acid Hemicalcium Salt (Supelco)

PEG 3350 (Sigma-Aldrich)

Phosphoric Acid (Sigma-Aldrich)

Pyridoxine Hydrochloride (Sigma)

Quinine (Sigma)

Rapid DNA Ligation Kit (Sigma-Aldrich)

Restriction Endonucleases (HindIII, XhoI, XmaI, SacII) (New England Biolabs)

Salmon Sperm Deoxyribonucleic Acid (Sigma-Aldrich)

QIAprep Spin Miniprep Kit (Qiagen)

TAE Buffer (Amresco)

TE Buffer (USB)

Thiamin (Sigma)
Tris (Base) (JT Baker)

Tryptone (Sigma)

TX-100 (Sigma-Aldrich)

Yeast Extract (BD)

Yeast Nitrogen Base without Amino Acids (BD)

YPD (Yeast Extract Peptone Dextrose) Broth (BD)

ZnSO₄ (Fisher)

2.2. Yeast Strain

The trk1Δtrk2Δ mutant S. cerevisiae strain used in this study was SGY1528 (W303, MATa ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 trk1::HIS3 trk2::TRP1; kindly provided by Dr. Minor lab) (Tang et al., 1995). It was constructed by partly removing TRK1 and TRK2 coding regions and inserting non-functional allele, trk1::HIS3 and trk2::TRP1 into the TRK1 and TRK2 locus, respectively, which resulted in the lack of K⁺ uptake system and the inability to grow under low [K⁺]ₒ conditions. SGY1528 cells were stored in 15% glycerol solution at -80°C (Amberg et al., 2005).

2.3. Molecular Biology

The vector used in this study was a modified pYES2 plasmid, pYES2-MET25, with the inducible MET25 promoter (kindly provided by Dr. Minor lab) (Figure 7) (Minor et al., 1999). pYES2-MET25 with a high-copy-number gets access to replicate in both yeast and bacteria cells (2 μ and
Ori). The ampicillin resistance gene (AmpR) allows antibiotic selection in *E. coli* and *CYC1* transcriptional terminator helps efficient termination of mRNA. The plasmid also contains *URA3* gene substituting for the demand of uracil, which is used to select the transformants with the *URA3* mutant genotype. *MET25* promoter is introduced to initial the transcriptional expression in the absence of methionine, while to be repressed in the presence of methionine (Minor *et al.*, 1999).

pYES2-MET25 plasmids carrying sequences encoding TREK-1 (pYES2-MET25-mK2P2), Trk1 (pYES2-MET25-TRK1), Kir2.1 (pYES2-MET25-Kir2.1) and Kir2.1 mutant (pYES2-MET25-Kir2.1-H5-Stuffer) were kindly provided by Dr. Minor lab (Minor *et al.*, 1999; Chatelain *et al.*, 2005; Bagriantsev *et al.*, 2011). The coding sequences of hTWIK-1, rTWIK-1-T118I (both TWIK-1 and TWIK-1-T118I are referred to TWIK-1-K274E and TWIK-1-K274E-T118I in the rest of this paper), rTWIK-2 and rTWIK-3 were cut from distinct plasmids with varied pairs of restriction enzymes (Figure 8), gel purified by QIAquick Gel Extraction Kit, and subcloned into pYES2-MET25, respectively, by Rapid DNA Ligation Kit. The constructed plasmids were amplified and isolated from *E. coli, S. cerevisiae* SGY1528 and *E. coli* (XL1-Blue Subcloning-Grade Competent Cells, from Agilent Technologies), subsequently by QIAprep Spin Miniprep Kit, and verified by DNA sequencing (Table 1; Functional Biosciences, Inc.). Plasmid extraction from yeast was also supplemented with acid-washed glass beads (from Sigma-Aldrich). All procedures were performed using standard molecular biology techniques.
Figure 7. The map of pYES2-MET25 vector.

pYES2-MET25 contains the sequence of origins of replication for both yeast and bacterial (2 \( \mu \) and Ori, respectively). AmpR represents the Ampicillin resistance gene for bacterial selection marker, while URA3 is for yeast selection marker. CYC1 represents the transcriptional terminator. The K\(^+\) channel gene of interest is under control of the MET25 promoter. Modified from (Bagriantsev & Minor, 2013b).
Figure 8. A scheme of pYES2-MET25 plasmid construction.

(A) pYES2-MET25-hK2P1: human KCNK1 within a 1289-bp fragment was cut from pRAT-GFP-hK2P1 with HindIII and XhoI and was subcloned into the vector pYES2-MET25 after digesting pYES2-MET25-K2P2 with the same restriction enzymes, HindIII and XhoI. (B) pYES2-MET25-rK2P1-T118I: rat KCNK1-T118I was cut from pMAX-rK2P1-T118I within a 1605-bp fragment flanked by Xmal and XhoI sites, and then subcloned into
the same sites of the vector backbone after digesting pYES2-MET25-mK2P2 with Xmal and Xhol. (C) pYES2-MET25-rK2P6: rat KCNK6 was cut with HindIII and Xhol from pcDNA3.1(+)rK2P6, within a 954-bp fragment and was subcloned into vector pYES2-MET25 after digesting pYES2-MET25-K2P2 with HindIII and Xhol. (D) pYES2-MET25-rK2P7: rat KCNK7 was cut from pRAT-rK2P7 within a 1054-bp fragment using HindIII and SacII and was then subcloned into the vector pYES2-MET25 after digesting pYES2-MET25-hK2P1 with HindIII and SacII.
Table 1. Sequencing primers designed for transformation confirmation

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Sequence</th>
</tr>
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<tr>
<td>pYES2-MET25-mK2P2 Forward</td>
<td>5’-GATCAGGGTAGAGGACGACCAC-3’</td>
</tr>
<tr>
<td>pYES2-MET25-mK2P2 Reverse</td>
<td>5’-CAAGTTCCAGCGTGCCACA-3’</td>
</tr>
<tr>
<td>pYES2-MET25-TRK1 Forward</td>
<td>5’-TCCTTTTTCCGTTAGAGCGG-3’</td>
</tr>
<tr>
<td>pYES2-MET25-TRK1 Reverse</td>
<td>5’-CTTCGTGTAATAACAGGGT-3’</td>
</tr>
<tr>
<td>pYES2-MET25-hK2P1 Forward</td>
<td>5’-TCCTTTTTCCGTTAGAGCGG-3’</td>
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<tr>
<td>pYES2-MET25-hK2P1 Reverse</td>
<td>5’-CTTCGTGTAATAACAGGGT-3’</td>
</tr>
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<td>5’-TCCTTTTTCCGTTAGAGCGG-3’</td>
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<tr>
<td>pYES2-MET25-rK2P1-T118I Reverse</td>
<td>5’-CTTCGTGTAATAACAGGGT-3’</td>
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<tr>
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</tr>
<tr>
<td>pYES2-MET25-rK2P7 Forward</td>
<td>5’-TCCTTTTTCCGTTAGAGCGG-3’</td>
</tr>
<tr>
<td>pYES2-MET25-rK2P7 Reverse</td>
<td>5’-CTTCGTGTAATAACAGGGT-3’</td>
</tr>
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</table>
2.4. Yeast Culture and Media

All mediums were made following previous procedures (Bagriantsev & Minor, 2013b; Cosentino et al., 2017) with slight modifications on component concentrations.

SGY1528 yeast culture was grown at 30°C in rich YPAD medium (50 g/L YPD broth, 24 mg/L adenine hemisulfate; 20 g/L agar for solid medium) supplemented with 100 mM KCl (YPAD 100K).

SGY1528 yeast strains carrying a plasmid were grown at 30°C in synthetic dropout medium without uracil and methionine (6.7 g/L yeast nitrogen base, 20 g/L glucose, 0.73 g/L CSM-Met-Ura powder, 0.12 g/L glutamic acid, 40 mg/L adenine hemisulfate; pH 6.5 adjusted by 1 M Tris base) supplemented with 100 mM KCl (-Ura/-Met 100K). Plates were made following the above recipe with the addition of 20 g/L agar.

The selective medium (-Ura/-Met 1K) was used to select transformed SGY1528 expressing functional $K^+$ channels (0.73 g/L CSM-Met-Ura powder, 0.12 g/L l-glutamic acid, 1% glucose, 2.1 g/L arginine, 40 mg/L adenine hemisulfate, 1x trace minerals solution (described below), 1x vitamin solution (described below), 1 mM MgSO$_4$, 0.1 mM CaCl$_2$, 1 mM KCl, and pH 6.0 adjusted by phosphoric acid). Plates were made with the addition of 15 g/L agarose LE with low [$K^+$]$_o$.

Vitamin 1000X stock solution was made by mixing 2 mg/L biotin, 400 mg/L pantothenic acid, 400 mg/L pyridoxine, 400 mg/L thiamin and 2 g/L inositol in 1 L water. Trace mineral 1000X stock solution was made by mixing 50 mg/L boric acid, 4 mg/L CuSO$_4$, 10 mg/L KI, 50 mg/L FeCl$_3$, 45 mg/L MnSO$_4$, 100 mg/L molybdic acid, 70 mg/L ZnSO$_4$, and 1 ml concentrated HCl in 1 L water. Stock solutions were sterilized through 0.22-$\mu$m bottle-top vacuum filters (from CORNING) and stored at 4°C.
2.5. Yeast Transformation

Yeast cells were transformed with plasmids using the lithium-chloride method (Bagriantsev et al., 2013): 5 mL of yeast SGY1528 culture in stationary phase was pelleted and resuspended with 100 µL Li-TE (100 mM LiCl, 10 mM Tris 7.5, 1 mM EDTA), 200 µL PEG-TE (50% w/v PEG-3350, 10 mM Tris 7.5, 1 mM EDTA), 100 µg salmon sperm carrier DNA, and approximately 2 µg of plasmid DNA (pYES2-MET25-mTREK1, pYES2-MET25-TRK1, pYES2-MET25-Kir2.1, pYES2-MET25-Kir2.1-H5-Stuffer, pYES2-MET25-hTWIK-1, pYES2-MET25-rTWIK-1-T118I, pYES2-MET25-rTWIK-2 or pYES2-MET25-rTWIK-3); mixtures were heat-shocked at 42°C for 10 minutes, rinsed, and cultivated at 30°C in -Ura/-Met 100K plate for 3 days.

Transformed yeasts were selected from -Ura/-Met 100K plates, amplified in 2X YPAD 100K medium, and stored in frozen competent cell solution (5 % v/v glycerol, 10 % v/v DMSO) at -80°C (Gietz & Schiestl, 2007).

2.6. Yeast Rescued Growth

SGY1528 yeasts with or without heterologous expression of a type of K⁺ channels were inoculated in the rich YPAD 100K medium and grew to stationary phase. Cultures were then washed, diluted to equivalent OD₆₀₀ (optical density at 600 nm), and streaked onto YPAD 100K plates, -Ura/-Met 100K plates and -Ura/-Met 1K plates, sequentially, with 2-7 days incubation at 30°C. Plates were photographed using a BioRad Chemidoc XRS/MP Imager and Image Lab software. For liquid cultures, transformed yeast cells were incubated in -Ura/-Met 100K medium until the OD₆₀₀ reached 0.3-0.8, while control yeast cells were incubated in the YPAD 100K medium. Cells were then washed and resuspended with -Ura/-Met 1K to an OD₆₀₀ of 0.25. In the following 48 hours, cells were incubated at 30°C with constant shaking. At the indicated time points (0, 4, 8, 12, 20, 28, 36, 48 hours), 200 µL cultures were collected and diluted by a factor of 1:10, and their OD₆₀₀
were measured via Lambda-35 UV/Vis Spectrophotometer and a 10mm-path-length quartz cuvette (from Starna Cells).

2.7. Disc Diffusion Assay on Agarose Plates

The disc diffusion assay was carried out as described previously (Fernandez-Acero et al., 2012; Bagriantsev & Minor, 2013b). Transformed yeasts carrying functional K\(^+\) channels were incubated in -Ura/-Met 100K until saturated. The cultures were then washed and resuspended with -Ura/-Met 1K to an OD\(_{600}\) of 0.01 of TWIK-1, TWIK-2, and TWIK-3 cultures, an OD\(_{600}\) of 0.05 of TREK-1 culture and an OD\(_{600}\) of 0.1 of Kir2.1 culture. Diluted cellular suspensions were spread over the surface of -Ura/-Met 1K agarose plates with a sterile swab (from Puritan). Then 10 μL of different concentrations of BaCl\(_2\) (1 mM, 5 mM, 10 mM), quinine (1 mM, 5 mM, 10 mM) or TX-100 (0.005%, 0.05%, 0.5%) solution was spotted onto the 6 mm sterile paper discs (from BD) on top of the plates. All drug solutions were sterilized through 0.22 μm filters. During the following 7-10 days’ 30°C incubation, 5 μL of drug solutions with the same concentration was added to each disc every day. Then the photographs of the plates and diameter or radius of the inhibition zone surrounding each disc were collected.

2.8. TX-100 Inhibition Test in Liquid Culture Medium

SGY1528 yeasts expressing TWIK-1 channels were incubated in -Ura/-Met 100K until the OD\(_{600}\) reached 0.3-0.8. Then the cultures were resuspended to an OD\(_{600}\) of 0.25 with -Ura/-Met 1K and supplemented with different concentrations of TX-100 (0.005%, 0.05%, 0.5%), and incubated at 30°C with constant shaking. The OD\(_{600}\) values with a dilution of 1:10 were measured at the indicated time points (0, 4, 8, 12 hours).
2.9. Statistical Analysis

All data were analyzed using Origin statistical software. The results are presented as the mean ± SEM with at least 3 independent cultures. Representative plate images were obtained from at least 3 biological replicates by BioRad Chemidoc XRS/MP Imager. The growth curves were generated by nonlinear regression analysis to fit a Gompertz equation. Statistical significance was assessed via two-way ANOVA test. Assessments with p < 0.05 were considered significant.
3 Rescue Growth of K⁺-Uptake–Deficient Yeasts by Heterologous Expression of TWIK Channels

3.1. Expression of K⁺ Channels Rescues Growth of K⁺-uptake-deficient SGY1528 Yeasts

As a yeast strain deficient in K⁺ uptake, SGY1528 lack K⁺ transporters and cannot survive under low [K⁺]₀ (0.5 – 10 mM) conditions (Grishin et al., 2006; Cioffi et al., 2015), though SGY1528 yeasts are able to grow under high [K⁺]₀ (100 mM) (Figure 9B) (Sadja et al., 2001) depending on nonselective transporters, like Tok1, Ena1, Nha1 and Pho89 (Arino et al., 2010; Yenush, 2016). Heterologous background K⁺ channels functionally replace yeast K⁺ transporters and restore the growth under low [K⁺]₀ conditions.

To express K⁺ channels in mutant yeast strains, the TWIK coding regions were subcloned into a dual-expression vector pYES2-Met25, which was subsequently transformed to SGY1528 using a lithium-chloride method. Transformants that grew on the synthetic dropout plates supplied with 100 mM KCl (-Ura/-Met 100K) were selected for following experiments. In the dropout medium, the lack of uracil selected for the URA3 mutant yeast containing the plasmids. While the absence of methionine drove the transcriptional expression of K⁺ channel from the plasmids. After culturing for 3 days, yeast cells carrying the plasmids with URA3 gene grew under uracil-absent condition (Figure 9B, C; Figure 10B). Since the concentrations of amino acid and nutrition components are not similar in the study, the low [K⁺]₀ medium formulation differed slightly from previous ones (Bagriantsev & Minor, 2013a; Cosentino et al., 2017). As a result, it is necessary to first verify whether the adjusted growth supplements support the rescued growth. It has been reported that expressing functional K⁺ channel Kir2.1 and TREK-1 in SGY1528 can produce K⁺
uptake and restore the growth under low $[K^+]_o$ condition (Bagriantsev et al., 2011; Gebhardt et al., 2011). Kir2.1 mutant $K^+$ channel is made by cloning a non-functional ‘stuffer sequence’ into the pore-forming H5 region, which eliminates its pore activity (Minor et al., 1999). Expressing Kir2.1 mutant $K^+$ channels in SGY1528 had no effect on its inability to grow under low $[K^+]_o$ condition (Bagriantsev et al., 2011). SGY1528 yeast strains were transformed with plasmids encoding Trk1, TREK-1, Kir2.1, or Kir2.1 mutant $K^+$ channels. Once grown on the -Ura/-Met 100K medium, the colonies were transferred to a selective medium containing 1 mM KCl (-Ura/-Met 1K), which served as a low $[K^+]_o$ environment. The low $[K^+]_o$ plates were incubated for 7 days until the yeast colonies grew. Trk1, as the yeast primary $K^+$ transporter, restored yeast growth under low $[K^+]_o$ condition as expected (Figure 9D). Furthermore, yeast expressing functional $K^+$ channel Kir2.1 (Figure 9D) and TREK-1 (Figure 10C) regained the ability to uptake $K^+$ and grew under low $[K^+]_o$ condition, consistent with previous results (Bagriantsev et al., 2011; Gebhardt et al., 2011). By expressing Kir2.1 mutant $K^+$ channel in the yeast cell strains, cells were unable to grow under low $[K^+]_o$ condition (Figure 9D). The results indicate that this adjusted supplemental medium makes access to detect the rescue activities of functional $K^+$ channels as they enable $K^+$ uptake for the $K^+$-uptake-deficient strain under low $[K^+]_o$ condition.
Figure 9. $K^+$-uptake-deficient SGY1528 yeasts, which heterologously express TWIK-1 channels, restore to grow under low [$K^+]_o$ condition.

(A) Each agar plate was separated into six sectors of indicated $K^+$ channels expressed in SGY1528; control is yeast alone without transforming any plasmids. (B) Yeast growth on rich YPAD medium containing 100 mM KCl. YPAD 100K plates were incubated at 30°C for 2 days. (C) Synthetic -Ura/-Met dropout medium selected transformants that had taken up plasmids. Plates were incubated at 30°C for 3 days. (D) Yeast was rescued by functional $K^+$ channels on -Ura/-Met low [$K^+]_o$ medium (1 mM KCl). Plates were incubated at 30°C for six days. All yeasts were inoculated in liquid -Ura/-Met 100K and streaked onto indicated plates with diluting to same cell optical density. n = 3.
3.2. Heterologous Expression of TWIK Channels Rescues Growth of K⁺-uptake-deficient Yeast on Agar Plates with Selective Medium

To investigate whether expression of TWIK channels rescues growth of SGY1528 yeasts in low [K⁺]₀, TWIK-1, TWIK-2, or TWIK-3 in pYES2-Met25 vectors, respectively, were transformed into SGY1528. After culturing on -Ura/-Met 1K plates for 7 days, SGY1528 expressing each TWIK channel grew well on solid media at low [K⁺]₀ condition (Figure 9D; Figure 10C), indicating that TWIK channels enable SGY1528 mutant strains to uptake K⁺, consistent with the electrophysiological studies that TWIK-1 and TWIK-2 conduct inward K⁺ currents under hyperpolarized condition. Importantly, it was found that TWIK-3 channels also enabled K⁺ influx into SGY1528 yeasts, which for the first time suggests that TWIK-3 channels are functional channels.

TWIK-1 K⁺ channels are sensitive to extracellular acidification; thus, the K⁺-uptake-deficient strain needs to grow in a lowly acidic condition (Navarrete et al., 2010). To eliminate the effect of acidification of the medium generated during yeast growth, TWIK-1-T118I K⁺ channels were expressed in SGY1528 and the growths were measured. TWIK-1-T118I channels have a mutant at the Thr118 residue near the selectivity filter of TWIK-1 and perform different physiological characteristics compared to TWIK-1 under subphysiological [K⁺]₀ condition. It abolishes the reduced K⁺ selectivity of TWIK-1 K⁺ channels in both neutral and acidic environments (Ma et al., 2011a; Chen et al., 2014a). The yeast expressing TWIK-1-T118I that grew in Ura/-Met 1K showed no significant growth difference compared to the rescued yeast expressing TWIK-1 (Figure 9D, Figure 11F), indicating that the rescued growth dependent on TWIK-1 or TWIK-1-T118I was not affected by the extracellular pH of the solid media.
Figure 10. TWIK-2 and TWIK-3 functionally rescue yeast deficient in K$^+$ uptake under low [K$^+$]. condition.

(A) The physical growth position of indicated K$^+$ channels expressed in SGY1528 on agar plates. (B) Plasmid selective growth on -Ura/-Met 100K plates with incubation at 30°C for 2-3 days. (C) Rescued growth on -Ura/-Met 1K plates with incubation at 30°C for 6 days. Yeasts were inoculated in liquid -Ura/-Met 100K and streaked onto indicated plates with diluting to same cell optical density. n = 3.
3.3. Heterologous Expression of TWIK Channels Rescues Growth of K⁺-Uptake-Deficient SGY1528 Yeasts on Liquid Selective Medium

Solid culture can provide reliable results, but for screening purposes, liquid culture raises efficiency since the procedure is simple and fast. To further explore if the viability of the rescue system and rescued growth patterns in liquid media are the same as in solid media, the rescued yeast culture densities were monitored in liquid medium under low [K⁺]₀ condition. SGY1528 yeasts showed slight growth in the first 12 hours and decreased growth over the following 36 hours (Figure 11A), confirming that the K⁺-uptake-deficient SGY1528 yeast cells could not survive under low [K⁺]₀ condition.

Trk1 is the primary yeast K⁺ transporter which resumes the growth of mutant yeast and leads to optimal rescued growth (Bagriantsev et al., 2013). The cultures of Trk1-expressing yeasts were monitored for 120 hours. It remained a high growth rate during the first 48 hours and entered stationary phase at 48 hours (Figure 11B). Thus, the cell density within 48 hours were also monitored for cultures that expressing other K⁺ channels. All yeast cultures expressing a TWIK K⁺ channel displayed growth within 48 hours (Figure 11E, F, G, H), indicating that TWIKs functionally rescued growth of K⁺-uptake-deficient SGY1528 yeasts in liquid media (Figure 11E, G, H). Yeasts expressing TREK-1 (Figure 11D), TWIK-1-T118I (Figure 11F), and TWIK-2 (Figure 11G) showed similar growth patterns. They underwent a logarithmic growth phase and reached the stationary phase 36 hours incubation-period (Table 2). However, the yeasts expressing Kir2.1 had an exponential growth within 48 hours and a higher maximum density (Table 2; maximum density not detected). Exponential growths were also observed in the TWIK-1 and TWIK-3-expressing yeast cultures. The results indicated that both Kir2.1 and K₂P K⁺ channels display their function in K⁺ uptake. Yeasts expressing different K⁺ channels also have different
rescue growth patterns due to their growth rate and maximum cell density (Table 2). Interestingly, TWIK-3-expressing yeasts retained a similar growth rate as TREK-1-expressing yeasts, and had a similar growth pattern with TWIK-1-expressing yeasts, indicating that TWIK-3 channels functionally uptake $K^+$ but may have a different $K^+$ selectivity compared to other TWIKs.
**Figure 11. Growth of K⁺-uptake-deficient yeasts expressing indicated K₂P channels or transporters.**

K⁺-uptake-deficient yeast SGY1528 were transformed with K⁺ channels or transporters including (B) Trk1, (C) Kir2.1, (D) TREK-1, (E) TWIK-1, (F) TWIK-1-T118, (G) TWIK-2, or (H) TWIK-3, and grew on selective -Ura/-Met 1K medium. Yeast strain SGY1528 was grown as negative control (A). Yeast strain and transformants were first incubated in liquid -Ura/-Met 100K and diluted to same starting OD₆₀₀ (optical density at 600 nm) in -Ura/-Met 1K. Cell cultures were incubated at 30°C with vigorous shaking. OD₆₀₀ was measured at indicated time points. Curves were generated by nonlinear regression analysis to fit a Gompertz equation. Error bars indicate SEM. n = 3-8 independent cultures.
Table 2. Growth curve parameters of yeast cultures expressing different K$^+$ channels.

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<tr>
<th></th>
<th>Control</th>
<th>Trk1</th>
<th>Kir2.1</th>
<th>TREK-1</th>
<th>TWIK-1</th>
<th>TWIK-1-T118I</th>
<th>TWIK-2</th>
<th>TWIK-3</th>
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<tr>
<td>Starting Density</td>
<td>0.2382</td>
<td>0.2908</td>
<td>0.4753</td>
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<td>8.644</td>
<td>15.56</td>
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4 EXAMINATION OF THE YEAST RESCUE SYSTEM WITH K+ CHANNEL BLOCKERS

4.1. Growth of SGY1528 Yeasts Expressing Kir2.1 Channels is inhibited by Ba2+

To determine whether this rescue system is capable of detecting inhibitory activities of the blockers, a disc diffusion assay was adopted. It is quantified by measuring the diameters of inhibition or exhibition zones (Bacharach & Cuthbertson, 1948). Here, because the rescued growth either in solid or liquid media is dependent upon the function of K+ channel expressed in SGY1528 yeasts, if any chemical or drug applied has an inhibitory effect on the K+ channel, it will produce a zone of inhibition on the plates. To provide a uniform the colony number for each culture, the rescued yeast cultures were diluted and cultures with different OD600 were spread on -Ura/-Met 1K plates. After plating, the highest OD600 for each culture that had separated colonies were selected for the following inhibition experiments.

Growth of SGY1528 yeasts expressing Kir2.1 channels has been proved to be inhibited by Ba2+ (Chatelain et al., 2005; Bagriantsev et al., 2011). It has been also reported previously that TREK-1 is inhibited by 8 mM Ba2+ under low [K+]o condition (Bagriantsev et al., 2011). Therefore, yeast expressing Kir2.1 or TREK-1 was used to examine the applicability of this modified rescue system. Application of 1 mM Ba2+ caused a diameter of 27.55 ± 14.59 mm inhibition zones in plates with SGY1528 yeasts expressing Kir2.1 channels (p<0.05), 5mM Ba2+ induced inhibition zones with a diameter of 40.30 ± 8.02 mm (p<0.01), and 10 mM Ba2+ resulted in diameter of 48.09 ± 14.95 mm inhibition zones (p<0.01) (Figure 12A, B). However, 1 mM, 5mM, and 10 mM Ba2+ did not affect the rescued growth of TREK-1-expressing yeast. It might be explained by low sensitivity of
TREK-2 to Ba\textsuperscript{2+}.

4.2. Effects of Ba\textsuperscript{2+}, Quinine, and TX-100 on Growth of SGY1528 Yeasts That Express TWIK Channels on Agar Plates

Growth of yeast expressing Kir2.1 has been shown to be inhibited by Ba\textsuperscript{2+}, indicating that inhibitory effects can be detected in the system. To further investigate if TWIK channels can be inhibited by K\textsuperscript{+} channel inhibitors, Ba\textsuperscript{2+} and quinine were tested by disc diffusion assay. However, there was no inhibition zone on agar plates with SGY1528 yeasts expressing each TWIK after application of 1 mM, 5 mM, and 10 mM Ba\textsuperscript{2+} or quinine (Figure 13B, C; Figure 14B, C; Figure 15B, C). TX-100 was also tested in this study. It has been reported to inhibit Kir2.1 inward K\textsuperscript{+} currents (Hollerer-Beitz and Heinemann, 1998), implying that TWIKs can be also affected by TX-100. Interestingly, the agar plates applied with 0.5\% TX-100 formed unique ring-shaped zones around the paper discs. In the agar plates, growing SGY1528 yeasts that express TWIK-1, a normal growth zone was formed within zones of a diameter of 23.68 ± 2.96 (n=4) mm but there was no growth between 23.68 ± 2.96 (n=4) mm and 56.45 ± 5.03 (n=4) mm annular zone (Figure 13D). On agar plates cultured with SGY1528 yeasts expressing TWIK-2, normal colonies grew inside zones with a diameter of 31.81 ± 8.00 (n=3) mm and outside zones with a diameter of 52.37 ± 11.74 (n=3) mm around the paper disc containing 0.5\% TX-100. While in the 31.81 ± 8.00 (n=3) mm – 52.37 ± 11.74 (n=3) mm annulus, some colonies still grew, but they were smaller and lighter than the colonies from the rest of the plates (Figure 14D). In the agar plates seeded with SGY1528 yeasts expressing TWIK-3, normal growth also occurred in zones with a diameter of 29.10 ± 7.48 (n=4) mm zone, and there was almost no growth in the annular region of 29.10 ± 7.48 (n=4) mm to 55.66 ± 11.63 mm (Figure 15D). In addition, a higher density of yeast cells expressing TWIK in each plate, 0.05\% TX-100 also generated the same ring-shaped zones around the paper discs for
all three TWIKs. And when treated the yeast expressing either Kir2.1 or TREK-1 K⁺ channel with 0.5% and 0.05% TX-100, there were no effects detected in the plates (Figure 16). It is implicated that there might be specific activities that interacted between TX-100 and TWIK K⁺ channels.

4.3. Effects of TX-100 on Growth of SGY1528 Yeasts That Express TWIK-1 channels in Liquid Culture Media

Due to that TX-100 induced a ring-shaped inhibition zone on agar plates cultured with SGY1528 yeasts expressing TWIK channels, the inhibitory activities of TX-100 on growth of TWIK-1-expressing SGY1528 yeasts were further investigated in the liquid culture medium. Both 0.5% and 0.05% TX-100 significantly inhibited growth of the yeasts in cultures for 12 hours (Figure 17, p < 0.05), which are consistent with results in solid assay under 0.5% and 0.05% TX-100. 0.0005% TX-100 also has a slightly decreasing trend on the growth after 8 hours (Figure 17).
Figure 12. Effects of Ba$^{2+}$ on growth of SGY1528 yeasts expressing Kir2.1 or TREK-1 channels with disc diffusion assay.

K$^+$-uptake-deficient yeast strain expressing Kir2.1 or TREK-1 was incubated in liquid -Ura/-Met 100K before spreading onto -Ura/-Met 1K plates. (A) 1 mM, 5 mM and 10 mM Ba$^{2+}$ solutions were added to the discs every incubation day with indicated concentrations. Sterile dH$_2$O (0 mM Ba$^{2+}$, control) and 10% bleach worked as the negative and positive controls, respectively. (B) Quantification of the inhibition effects of Ba$^{2+}$ on SGY2538
yeast expressing Kir2.1. The inhibition zones are measured by diameters of the area lacking yeast cell growth. Data are presented as mean ± SEM. * p < 0.05, compared to 0 mM Ba\(^{2+}\) control; ** p < 0.01, compared to 0 mM Ba\(^{2+}\) control. n = 3-4 biological replicates.
Figure 13. Effects of Ba$^{2+}$, quinine, and TX-100 on growth of SGY1528 yeasts expressing TWIK-1 channels.

Cultures expressing TWIK-1 were first incubated in liquid -Ura/-Met 100K and spread to -Ura/-Met 1K plates with certain OD$_{600}$ determined previously. (A) Sterile dH$_2$O and 10% bleach worked as negative and positive controls. (B) Ba$^{2+}$, (C) quinine, and (D) TX-100 at indicated concentrations were added to the paper discs on the -Ura/-Met 1mM K$^+$ plates. The bleach generated a diameter of 21.79 ± 9.44 mm inhibition zone as positive control. All displayed plates were made from the same culture, and all results were repeated triplicated. n = 3.
Figure 14. Effects of Ba^{2+}, quinine, and TX-100 on growth of SGY1528 yeasts expressing TWIK-2 channels.

Cultures expressing TWIK-2 were first incubated in liquid -Ura/-Met 100K and spread to -Ura/-Met 1K plates with certain OD_{600} determined previously. (A) Sterile dH_{2}O and bleach worked as negative and positive controls. (B) Ba^{2+}, (C) quinine, and (D) TX-100 at indicated concentrations were added to the paper discs on the -Ura/-Met 1mM K^{+} plates. The bleach generated a diameter of 22.40 ± 8.70 mm inhibition zone as positive control. All displayed plates were made from the same culture, and all results were repeated triplicated. n = 3.
Figure 15. Effects of Ba^{2+}, quinine, and TX-100 on growth of SGY1528 yeasts expressing TWIK-3 channels.

Cultures expressing TWIK-3 were first incubated in liquid -Ura/-Met 100K and spread to -Ura/-Met 1K plates with certain OD_{600} determined previously. (A) Sterile dH_{2}O and bleach worked as negative and positive controls. (B) Ba^{2+}, (C) quinine, and (D) TX-100 at indicated concentrations were added to the paper discs on the -Ura/-Met 1mM K^{+} plates. The bleach generated a diameter of 24.40 ± 10.80 mm inhibition zone as positive control. All displayed plates were made from the same culture, and all results were repeated triplicated. n = 3.
Figure 16. Effects of TX-100 on growth of SGY1528 yeasts expressing Kir2.1 or TREK-1 channels.

K⁺-uptake-deficient yeast strain expressing Kir2.1 or TREK-1 channels was incubated in liquid -Ura/-Met 100K before spreading onto -Ura/-Met 1K plates. 0.5% or 0.05% TX-100 was added to the discs every incubation day. Sterile dH₂O (control) and 10% bleach worked as the negative and positive controls, respectively. n = 1-3 biological replicates.
Figure 17. Effects of TX-100 on growth of SGY1528 yeasts expressing TWIK-1 channels in liquid culture medium.

SGY1528 yeasts expressing TWIK-1 were resuspended in -Ura/-Met 1K media supplemented with the indicated concentrations of TX-100. Cultures were incubated at 30°C with vigorous shaking for 12 hours. OD<sub>600</sub> (optical density at 600 nm) was measured at indicated time points. Error bars indicate SEM. * p < 0.05, compared to 0% TX-100 control cultures. n = 3 independent cultures.
5 DISCUSSION

Here I employed the $K^+$-uptake-deficient SGY1528 yeast, to construct the growth-rescued system by heterologous expression of $K_{\text{2p}}$ channels. Results indicated that growths of the SGY1528 yeast are rescued in both solid and liquid medium with low $[K^+]_o$ when Trk1, Kir2.1, or TREK-1 are heterologously expressed in the yeasts (Figure 9D, 10C, 11B, 11C, and 11D), consistent with previous studies (Bagriantsev et al., 2012). In contrast, nonfunctional Kir2.1 mutant $K^+$ channels did not rescue growths of the yeast cells under low $[K^+]_o$ condition (Figure 9D) (Minor et al., 1999; Bagriantsev et al., 2011). Secondly, $\text{Ba}^{2+}$, a potent Kir2.1 inhibitor, efficiently inhibited growths of Kir2.1-expressing SGY1528 yeast under low $[K^+]_o$ conditions in a dose-dependent manner (Figure 12A and 12B), consistent with previous results (Chatelain et al., 2005; Bagriantsev et al., 2011). However, $\text{Ba}^{2+}$ did not have any effects on TREK-1-expressing SGY1528 yeast (Figure 12A and 12B), as TREK-1 channels have a low sensitivity to $\text{Ba}^{2+}$ (Chatelain et al., 2005; Bagriantsev et al., 2011).

Heterologous expression of TWIK channels including TWIK-3 rescued the growth of $K^+$-uptake-deficient SGY1528 yeasts under low $[K^+]_o$ conditions (Figure 9D, 10C, 11E, 11G, and 11H). It is the first time to show that TWIK-3 channels play a functional role in conducting inward $K^+$ current. As $\text{Ba}^{2+}$ and quinine are not potent blockers on TWIK channels, they failed to inhibit the growth of SGY1528 yeasts expressing TWIK channels (Figure 13B, 13C, 14B, 14C, 15B, and 15C). However, TX-100 remarkably affected the growth of these yeasts in low $[K^+]_o$ conditions (Figure 13D, 14D, 15D, and 17). These results suggest that SGY1528 yeasts heterologously expressing TWIK channels provide an approach to screen inhibitors of TWIK channels.
5.1. Expression of TWIK Channels Rescue Growth of SGY1528 Yeast

Considering large K⁺ gradients across the cell membrane of yeasts, K⁺-uptake-deficient yeasts are widely used to study inward-rectifying K⁺ channels (Bañuelos et al., 1995; Uozumi et al., 1995; Grishin et al., 2006; Paynter et al., 2010a; Paynter et al., 2010b), hyperpolarization-activated K⁺ channels (Sesti et al., 2003), and other K⁺ channels that conducting inward currents (Balss et al., 2008; Paynter et al., 2008; Schwarzer et al., 2008; Gazzarrini et al., 2009; Greiner et al., 2011; Cosentino et al., 2015). K⁺-uptake-deficient yeasts were used to identify modulators for a K⁺ channel from a library of chemicals (Zaks-Makhina et al., 2004; Bagriantsev et al., 2013). They were also used to study structure and function of K⁺ channels by screening of gain-of-function mutant channels with random mutagenesis (Yi et al., 2001; Bichet et al., 2004; Chatelain et al., 2005; Shikano et al., 2005; Hertel et al., 2010; Gebhardt et al., 2012; Cioffi et al., 2015).

SGY1528 yeasts that Trk1 and Trk2 transporters are deleted are K⁺-uptake-deficient. They grow only in mediums with high [K⁺]₀ but not in mediums with low [K⁺]₀ such as 1 mM. Since we used the medium formulation differed slightly from the ones from other studies (Bagriantsev and Minor, 2013; Cosentino et al., 2017), we first verified that the adjusted mediums work the growth of the SGY1528 yeasts that heterologously express background K⁺ channels. Previous studies demonstrated that expression of Trk1 (Chatelain et al., 2005; Balss et al., 2008; Bagriantsev et al., 2011), Kir2.1 (Zerangue et al., 1999; Graves and Tinker, 2000; Sadja et al., 2001; Shikano et al., 2005; Bagriantsev et al., 2011), and TREK-1 (Bagriantsev et al., 2011) rescued the growth of SGY1528 yeasts in the selective medium with 0.5 – 7 mM [K⁺]₀. We confirmed that the expression of Trk1, Kir2.1, or TREK-1 restored the growth of SGY1528 yeasts under low [K⁺]₀ condition (Figure 9D, 10C, and 11B-11D). These results indicate that slightly-modified mediums work for the rescued SGY1528 yeasts system in both solid and liquid media.
5.2. TWIK-3 Channels Form Functional Channels when Expressed in Yeast

Although 15 mammalian $KCNK$ genes are identified, some $K_{2p}$ isoforms such as TWIK-3 channels do not produce detectable currents when expressed in mammalian heterologous systems (Salinas et al., 1999; Bobak et al., 2017, Bichet et al., 2015). TWIK-3 channels are highly expressed in the brain, heart, and lung (Lesage & Lazdunski, 2000; Medhurst et al., 2001). They are also found in cancer cells (Williams et al., 2013). TWIK-3 channels likely play physiological roles, but whether they are functional channels remains unknown. In this study, we showed that $K^+$-uptake-deficient SGY1528 yeasts, which heterologously expressed human TWIK-3 channels, restored normal growth curves in both agar plates and culture medium with 1 mM $[K^+]_o$ (Figure 10C and 11H), indicating that TWIK-3 channels are responsible for $K^+$ transport in such $K^+$-uptake-deficient yeasts in low $K^+]_o$ conditions. In positive controls, SGY1528 yeasts expressing TWIK-1 or Kir2.1 channels also have normal growth curves in 1 mM $[K^+]_o$ (Figure 11D, 11C, and 11E). In negative controls, $K^+$-uptake-deficient SGY1528 yeasts or SGY1528 yeasts expressing non-functional Kir2.1 mutant channels did not grow at all (Figure 9D). Therefore, TWIK-3 channels form functional channels when expressed in SGY1528 yeasts. We demonstrated functional TWIK-3 channels for the first time.

$K_{2p}$ channels including TWIK-1 and TWIK-2 channels generally have the GL(Y)G motif in the selective filter of the P2 domain (Lesage et al., 1996b; Salinas et al., 1999). TWIK-3 channels have an unusual GLE sequence in the selective filter of the P2 domain (Salinas et al., 1999), they can form functional channels and are responsible for $K^+$ transport in SGY1528 yeasts with expression of TWIK-3. They may have very small conductance when expressed in mammalian heterologous systems so that their currents could not be detected with whole-cell voltage-clamp techniques (Salinas et al., 1999; Bobak et al., 2017, Bichet et al., 2015). Human TWIK-1 channels
produced very small currents in mammalian heterologous systems such as CHO cells, while human TWIK-2 channels do not generate detectable currents. These results also indirectly support that TWIK-3 channels form function channels.

5.3. TX-100 is a Potential Inhibitor of TWIK K⁺ Channels

TX-100 is an amphiphilic detergent that has been observed to affect K⁺ channels (Hollerer-Beitz & Heinemann, 1998; Narang et al., 2013). By using the yeast-based rescue system, the ring-shaped zones were formed on the -Ura/-Met 1K agar plates in the presence with 0.5% TX-100 (Figure 13D, 14D, and 15D). TX-100 had inhibition effects on the growth of SGY1528 yeasts expressing TWIK-1 channels in liquid culture medium with 1 mM [K⁺]o in a dose-dependent manner (Figure 17). 0.5% and 0.05% TX-100 had significant inhibition activities in the liquid medium, similar to those observed in solid assay.

TX-100 may cause a non-monotonic dose response to SGY1528 yeasts expressing with either TWIK-1, TWIK-2, or TWIK-3 K⁺ channel. By treating the yeast expressing TWIK-1, TWIK-2 and TWIK-3 with TX-100 on the -Ura/-Met 1K agar plates by disc diffusion assay, it generated unique ring-shaped zones (Figure 13D, 14D, and 15D). The disc diffusion assay measures the diameter of inhibition zone to represent the ability of inhibitory effect of compounds (Finn, 1959). The inhibitor is first applied to the disc in the center on the plate, then diffused to whole plate. Thus, the concentrations of inhibitor are not homogeneous in the plate, but has a gradient change from the center to the edge of plates (Sykes and Rankin, 2014). The unique ring-shaped zones produced by TX-100 have a growth zone around the disc and an inhibition zone surrounded the growth zone. It implies that higher or lower concentration of TX-100 does not affect the TWIK-dependent rescued growth, but TX-100 within a range of concentrations inhibits TWIK function in uptaking K⁺ and inhibits the rescued yeast growth. Another possible explanation is that TX-100
at the higher concentration induces reversible permeabilization of cell membrane which may increase potassium influx. One study has found that 0.1% TX-100 triggers divalent cation influx, like Ca$^{2+}$, which may likely due to its effects on membrane permeabilization (Németh & Kurucz, 2005). In the negative control, 0.5% and 0.05% TX-100 did not have any inhibitory effects on growths of SGY1528 yeasts expressing either Kir2.1 or TREK-1 channels on agar plated with 1 mM [K$^+$]o. These results support that TX-100 may specifically inhibit TWIK channels.

5.4. Future Study

This yeast-based rescue system provides a potential approach for high-throughput screening of effects of chemical compounds on TWIK channels. Furthermore, to comprehensively study the function of TWIK channels in the yeast strain SGY1528, their electrophysiological characteristics also need to be determined. Some studies are using electrophysiological techniques directly on K$^+$-uptake-deficient yeast cells (Bertl et al., 1995; Roberts et al., 1999; Bertl et al., 2003). Due to the yeast cell wall and the small size of yeast cells, yeast protoplasts are used in patch-clamp to enlarge cell size and remove the effects of yeast cell walls on seal formation during patch-clamp (Bertl et al., 1998; Lemtiri-Chlieh & Ali, 2013; Terpitz et al., 2008). Future electrophysiological study can use this yeast protoplasts patch-clamp technique to further explore the roles of TWIK channels in the K$^+$-uptake-deficient yeast. Alternatively, patch-clamp techniques using Xenopus oocytes or mammalian cells are prevalently used in recent studies combined with the high-throughput screening using K$^+$-uptake-deficient yeast strain (Zerangue et al., 1999; Balss et al., 2008; Bagriantsev et al., 2011; Chatelain et al., 2005; Graves and Tinker, 2000; Sadja et al., 2001; Shikano et al., 2005;). It can provide an easier approach than yeast patch-clamp to study the pharmacological effects of TX-100 on TWIK-1 and TWIK-2.


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