Extracellular pH and high concentration of potassium regulate the primary necrosis in the yeast *Saccharomyces cerevisiae*.

Victoria Bidiuk
A N Bach Institute of Biochemistry RAS: Institut biohimii imeni A N Baha Rossijskoj akademii nauk
Alexander Alexandrov
A N Bach Institute of Biochemistry RAS: Institut biohimii imeni A N Baha Rossijskoj akademii nauk
Airat Valiakhmetov (airatv@ibpm.pushchino.ru)
Skryabin Institute of Biochemistry and Physiology of Microorganisms RAS: Institut biohimii i fiziologii mikroorganizmov imeni G K Skrabina RAN https://orcid.org/0000-0002-4739-0806

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Abstract

Extracellular pH has a significant impact on the physiology of the yeast cell, but its role in cell death has not been thoroughly investigated. We studied the effect of extracellular pH on the development of primary necrosis in Saccharomyces cerevisiae yeast under two general conditions leading to cell death. The first is sugar induced cell death (SICD), and the second is death caused by several specific gene deletions, which have been recently identified in a systematic screen. It was shown that in both cases, primary necrosis is suppressed at neutral pH. SICD was also inhibited by the protonophore dinitrophenol (DNP) and 150 mM extracellular K+, with the latter condition also benefiting survival of cell dying due to gene mutations. Thus, we show that neutral pH can suppress different types of primary necrosis. We suggest that changes to the cellular membrane potential can play a central role in yeast cell death.

Introduction

Fungi and yeast are capable of growing over a wide range of extracellular pH. In turn, the extracellular pH has a significant effect on the physiology of the fungal cell. It is known that extracellular pH, among other things, regulates gene expression (Peñalva and Arst 2002; Serrano et al. 2002; Penalva et al. 2008), ionic homeostasis (Ke et al. 2013), regulates a transition of the cytoplasm from a fluid- to a solid-like state (Munder et al. 2016) and controls the cell cycle (Hayashi et al. 1998).

Extracellular pH is involved in numerous physiological processes in higher eukaryotes. For example, cancer cells, mainly using the glycolytic pathway for the production of lactate, acidify the external environment, which leads to the induction of the expression of pro-metastatic factors (Webb et al. 2011; Kato et al. 2013). Conversely, alkalization of the extracellular environment is used to combat metastases (Hamaguchi et al. 2020). Also, acidic extracellular pH is involved in the regulation of cell death in cancer cells, switching it from apoptosis to necrosis (Meurette et al. 2005; Lan et al. 2007; Riemann et al. 2011). Cell death can take various forms which differ in various parameters, and the qualitative state of the plasma membrane is one of the most often tested parameters. If cell death is thought to occur due to plasma membrane permeabilization, this is termed primary necrosis (Carmona-Gutierrez et al. 2018). In primary necrosis, the rupture of the plasma membrane precedes (or occurs together with) the processes of disintegration of intracellular structures. During apoptosis, the destruction of intracellular structures occurs while maintaining an intact membrane. In this case, permeabilization of the plasma membrane can occur at the final stage of apoptosis (secondary necrosis). At the biochemical level, necrosis can happen without the activation of any enzymes or signaling pathways, while apoptosis always begins with the activation of caspases, followed by the irreversible activation of a number of intracellular mechanisms.

Cell death that has some resemblance to apoptosis in yeast cells was first reported in 1997 (Madeo et al. 1997) and has since attracted considerable attention. Necrosis was considered to be the result of a fatal exposure of the cell to an excessive amount of an apoptosis-inducing agent (Madeo et al. 1999; Ludovico

et al. 2001; Liang and Zhou 2007). External and internal triggers of cell death in yeast have been reviewed in (Falcone and Mazzoni 2016).

It has been shown that glucose, in the absence of other nutrients, induces apoptosis in stationary yeast (Granot et al. 2003). This phenomenon was termed glucose (sugar)-induced cell death (GICD or SICD). Later it was shown that SICD is independent of yeast metacaspase and dependent on reactive oxygen species (ROS) production (Hoeberichts et al. 2010). We recently reported on the necrotic nature of SICD in exponentially growing yeast (Valiakhmetov et al. 2019). In that work, we used ascorbate and glutathione to assess the effect of ROS on SICD. It is important to note that these scavengers must be neutralized to pH7 in order to be effective.

While SICD is an acute treatment which causes death of a considerable share of the cell population, primary necrosis has also recently been reported in the context of dividing cells harboring various deletions and down-regulating mutations in a large number of genes (Alexandrov A.I. et al., manuscript in preparation). This type of cell death, termed rapid division-associated necrosis, is chronic, was shown to be independent of ROS and is likely to be a case of primary necrosis.

In this work, we present data on the effect of extracellular pH and changes in membrane potential on SICD, as well as on increased cell death during division caused by several gene deletions.

Materials And Methods

Culture growth

Strains we used in the present study listed in Table 1. For SICD experiments the culture grew on the standard YPD medium (Applichem, FRG) for 15–17 h (mid exponential phase), was twice washed with distilled water and incubated in MilliQ water for 30 min at 28°C for complete depletion of glucose absorbed by cells and return of H^+-ATPase activity to a low, background level (Serrano 1983). Yeast cells were pelleted and suspended (1g 10 mL^{-1} w/w = 8 x10^8 cells/ml) in MilliQ water. For experiments on necrosis during cell division in mutants, cells were grown in YPD medium (or YPD medium with pH stabilized at 7) to logarithmic phase (OD_{600} = 0.3–0.6). YPD buffering was performed by titration with 2M NaOH to pH = 7, after which the medium was supplemented with 20mM phosphate buffer, pH = 7. Growth for testing of the effects of KCl was performed by growing cells in YPD that was supplemented with 150mM of KCl.

Table 1
Strains used in this study

Strain	Genotype	Source
SEY6210	MATα ura3-52 leu2-3,112 his3-Δ100 trp1-Δ901 lys2-801suc2-Δ9	(Robinson, et al. 1988: 4936-48)
SEY6210 rho^0	MATα ura3-52 leu2-3,112 his3-Δ100 trp1-Δ901 lys2-801suc2-Δ9 rho^0	This study
BY4741	MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0	Dharmacon
Δ*afo1*	BY4741 Δafo1	Dharmacon
Δ*ira2*	BY4741 Δira2	Dharmacon
Δ*rim21*	BY4741 Δrim21	Dharmacon
Δ*yca1*	BY4741 Δyca1	Dharmacon
Δ*yno1*	BY4741 Δyno1	Dharmacon
Δ*end3*	BY4742 Δend3	Euroscarf
Δ*scp1*	BY4742 Δscp1	Euroscarf
Δ*sla1*	BY4742 Δsla1	Euroscarf
ΔYPL102C	BY4741 ΔYPL102C	Dharmacon
Δpfd1	BY4741 Δpfd1	Dharmacon
Δsla1	BY4741 Δsla1	Dharmacon
Δpim1	BY4741 Δpim1	Dharmacon
ΔYBL094C	BY4741 ΔYBL094C	Dharmacon
Δcdh1	BY4741 Δcdh1	Dharmacon
Δaim44	BY4741 Δaim44	Dharmacon
Δvps1	BY4741 Δvps1	Dharmacon
ΔYJR011C	BY4741 ΔYJR011C	Dharmacon
Δpef1	BY4741 Δpef1	Dharmacon
Δmet7	BY4741 Δmet7	Dharmacon
Δiki3	BY4741 Δiki3	Dharmacon

Incubation with glucose

0.05 mL of 2M glucose in water was added to the tube with 0.95 mL of the cell suspension in water or buffer with different pH to study the effect of glucose. 0.05 mL of water was added to the control tube.

The samples were incubated in a ThermoMixer (Eppendorf, FRG) at 30°C and 1000 rpm.

Staining the cells and flow cytometry

For SICD experiments we used FITC-dextran (FD), m.w. 4 kDa (Sigma, USA) to estimate the membrane rupture. 1 µL of 1mM FD in water was added to 7 µL of the cell suspension to stain the cells with FD. The mixture was incubated at RT for 5 min, and 0.15 mL of water was added. The cells were pelleted at 13000 g for 30 secs, and the supernatant was discarded. The cells were washed two times more with 0.15 mL of water in the same manner, and the cell pellet was finally suspended in 0.15 mL water. The cells were immediately passed through a Flow Cytometer BD Accuri C6 (BD Biosciences, USA) and the fluorescence was detected using channel FL1 (488-nm excitation and 533-nm emission). 100 000 cells were counted at each experimental point. All assays were repeated 4–5 times and the mean results are presented.

For experiments on necrosis during cell division in mutants, cells were stained with propidium iodide (PI) (Sigma, USA) by direct addition to the cultivation medium (final concentration – 2 µg mL^{-1}, incubation time – 1 hour) and measured on the flow cytometer using channel FL2 (488-nm excitation and 585 nm emission).

1,2,3,-Dihydrorhodamine (DHR) staining assay

0.02 mL of 0.5 mg mL^{-1} 1,2,3,-dihydrorhodamine in DMSO (Sigma, USA) were added to 1 mL cell suspension in water. The samples were incubated in a ThermoMixer for 1 h at 30°C at 1000 rpm. The cells were pelleted at 13000 g for 1 min, and the supernatant was discarded. The cells were washed two times with 1 mL of water and the cell pellet was finally suspended in 0.95 mL water or buffer with different pH. 0.05 mL of 2M glucose in water was added to study the effect of glucose. 0.05 mL of water was added to the control tube. In 1 h 0.15 mL of water was added to 7 µL of cell suspension. Fluorescence was detected on flow cytometer using channel FL1 (488-nm excitation and 533-nm emission). 100 000 cells were counted at each experiment.

CFU assay

0,1 mL of SEY6210 cells after incubation for 1 h with 100mM glucose in water or in 50 mM HEPES pH 7 were plated on YPD-agar plates after the appropriate dilution. CFU numbers were counted after 48 h of growth at 28°C. Cells SEY6210 incubated in water were used as a control.

Determination of membrane potential by DiOC2(3) staining assay.

We used the fluorescent dye DiOC2(3) (ThermoFisher, USA) to register changes in the membrane potential under various conditions of cell incubation. We used the SEY 6210 rho^0 strain in order to exclude the contribution of the mitochondrial membrane potential. The strain was obtained by treating *S.cerevisiae SEY6210* cells with 0.1 mg mL^{-1} ethidium bromide overnight. Thereafter, cells were plated on YPD plates and colonies were tested for their ability to grow in ethanol. Were selected those colonies, whose cells did not show growth after 48 hours of incubation in YP medium + 1% ethanol. Cells were prepared as

described under *Culture growth* section (above). 0.05 mL of cells were added to 1 mL of MilliQ water or 1 mL of 50 mM HEPES pH 7.0. 0.1 mL of this cell suspension was plated in a 96-well black plate (Corning Costar, USA). Fluorescence was recorded on a FilterMax F5 plate reader (Molecular Devices, USA). We used wavelength 485 nm for excitation, and emission was recorded at 625 nm. Readings were taken every minute with 30 second shakings before each measurement. 1 µL of 3 mM DiOC2 (3) in DMSO was added to give a final dye concentration of 30 µM. The plate was incubated for 5 min at 30 ° C to distribute the dye. To initiate the process, 5 µL of 2M glucose was added to the wells.

Results

Stabilizing extracellular pH at 7 reduces SICD.

To assess the role of ROS in cellular processes, ROS scavengers such as glutathione, N-acetyl cysteine, and ascorbate are widely used. However, ascorbic acid and N-acetyl cysteine should be in neutralized form at pH 7 to avoid toxic effects on cells. As shown earlier, 10 mM ascorbate almost completely suppresses SICD (Valiakhmetov et al. 2019). Ascorbic acid (weak acid) neutralized with NaOH (strong base) to pH 7 is a buffer. In order to discriminate the effect of ascorbate as a scavenger for ROS from the buffer effect, we tested the effect of HEPES buffer on SICD and ROS generation upon incubation of *S. cerevisiae* yeast with glucose. Yeast metabolizing glucose in an unbuffered medium lowers the extracellular pH from 6 to 3.7 in 5 minutes and to 3.2 by the 60 minutes. (Fig. 1).

Incubation of SEY6210 cells with glucose in 50 mM HEPES pH7.0 buffer completely stops SICD and production of ROS. Suppression of ROS generation and SICD development has a clear pH relationship (Fig. 2).

As the external pH decreased, the number of cells with ROS and SICD increased. It should be noted that the chemical nature of the buffer was important. Good's buffers (HEPES, MES), due to their chemical inertness, were the best for pH 7, 6 and 5. For pH 4.2, glycine-glycine buffer was used. Non-Good's buffers used for pH 3.5 buffer (citrate, K-phthalate, Na-phthalate) showed non-specific effects on the ROS production and SICD (see below). Therefore, water adjusted to pH 3.5 with HCl was used to test the effect of the acidic external pH. The CFU test showed that after incubation of SEY6210 cells with glucose at pH 7.0, cell survival was 100%, and after incubation of cells in unbuffered media, it decreased by 18 ± 3%.

Since the extracellular pH influences the functioning of yeast cells in a variety of ways (Serra-Cardona et al. 2015) we tested the involvement of several cellular pathways known to be involved in pH-dependent signaling in the suppression of SICD by neutral pH. We used commercially available knockout mutants derived from the BY4741 parent strain (Table 1). The parental strain BY4741 was also tested for the development of SICD under our conditions (see Materials and Methods). The ROS generation and the development of SICD in the BY4741 strain were in good agreement with the data obtained earlier in the SEY6210 strain.

The Rim101/PacC pathway seemed the most likely responsible for the regulation of SICD by extracellular pH. The C-terminal region of Rim21 is known to be an ambient pH sensor (Nishino et al. 2015). However, *Δrim21* strain showed the same response in the neutral medium as the parent, i.e. absence of rim21p did not restore SICD at pH 7. The PKA pathway is involved in yeast response to alkaline stress (Casado et al. 2011). Deletion of *ira2* – small GTPase of Ras2 – did not remove sensitivity of SICD toward neutral pH.

Since SICD occurs in the presence of increased amounts of ROS, we tested the two main suppliers of ROS — the mitochondrial respiratory chain and NADPH oxidase of ER. Cells with increased resistance to oxidative stress *Δafo1* (Heeren et al. 2009) and *Δyno1* (Rinnerthaler et al. 2012) showed no SICD at pH 7. Deletion of the yeast metacaspase *yca1* also does not change the sensitivity of SICD to neutral pH. Our previous work also suggested that SICD occurs in a population of cells in the S-phase of the cell cycle (Valiakhmetov et al. 2019). It is known that actin dynamics (indirectly through the mitochondrial membrane potential) affects the generation of ROS (Gourlay et al. 2003; Gourlay et al. 2004; Gourlay and Ayscough 2005b). We tested the sensitivity of SICD to pH 7.0 in three knock-out mutants - *Δsla1*, *Δscp1* and *Δend3*, involved in regulation of actin dynamics (Gourlay et al. 2003). All three mutants showed no SICD at pH 7.0. As shown in Table 2, 50 mM HEPES pH 7.0 almost completely suppresses SICD in all the strains tested.

Table 2
Suppression of SICD and ROS generation in 50 mM HEPES pH 7.0 (Mean ± SD). Cells were incubated within 1 h either in water solution with 100 mM glucose, or 50 mM HEPES buffer (pH 7.0) with 100 mM glucose. Percentage of the dead cells were determined by the FD staining. Percentage of cell with high ROS content were determined by the DHR staining.

	SICD		ROS	
strain	**glucose**	**pH7**	**glucose**	**pH7**
SEY 6210	20 ± 4,9	0,47 ± 0,2	18 ± 2,36	2,8 ± 0,22
SEY 6210 rho^{-}	22 ± 4,3	0,9 ± 0,37	18,9 ± 1,8	1,4 ± 1,3
BY4741	19 ± 2,7	0,84 ± 0,31	17,9 ± 2,7	0,76 ± 0,49
Δafo1	21,5 ± 1,4	0,73 ± 0,22	22,8 ± 2,7	1,6 ± 0,68
Δend3	15 ± 1,4	0,87 ± 0,46	15,4 ± 3	0,86 ± 0,31
Δira2	17,5 ± 3,4	0,52 ± 0,12	19.8 ± 2,7	0,76 ± 0,3
Δrim21	23,3 ± 2,9	0,6 ± 0,12	21 ± 2,2	0,44 ± 0,11
Δscp1	15,1 ± 3,5	1,02 ± 0,25	15,7 ± 1,8	1,8 ± 1,5
Δsla1	25,2 ± 4,7	1,96 ± 0,14	18,5 ± 3,3	2,1 ± 0,96
Δyca1	13,4 ± 4,6	0,37 ± 0,15	14,5 ± 2	0,7 ± 0,27
Δyno1	15,2 ± 2,6	0,29 ± 0,05	17,3 ± 2,3	0,91 ± 0,43

Since we did not find a direct relationship between the functioning of the main signaling pathways and the suppression of SICD by neutral external pH, we hypothesized that the effect of pH might be mediated without the involvement of cellular signaling, but rather via some physic-chemical parameters of the cell. One such parameter is the membrane potential - $\Delta\mu H^{+}$. To test dependence of SICD on membrane potential, SEY6210 cells were incubated with glucose in unbuffered media in the presence of protonophore DNP. As can be seen from Fig. 3, DNP at a concentration of 0.5 mM suppresses SICD by more than 80%.

To assay the involvement of another component of $\Delta\mu H^{+}$ - $\Delta\Psi$, we tested the effect of 150 mM KCl on SICD. Exogenous K^{+} at a concentration of 150 mM suppressed ROS generation and SICD by 80% (Fig. 4).

In order to find out which of the 2 components $\Delta\mu H^{+}$ (ΔpH or $\Delta\Psi$) is responsible for the suppression of SICD by neutral external pH, we tracked the change in the membrane potential using the fluorescent dye DiOC2(3). To track changes in the membrane potential on the plasma membrane only and to exclude the contribution of the mitochondrial membrane potential, we used a SEY6210 rho^{0} strain. As can be seen

from Fig. 5, ΔΨ on the plasma membrane significantly increases during incubation of cells with glucose. At the same time, neutral extracellular pH or 150 mM KCl completely inhibit the increase in ΔΨ.

Thus, we can conclude that SICD depends on $\Delta\mu H^+$, with ΔΨ playing a larger role than ΔpH. with ΔΨ playing a larger role than ΔpH.

Effect of pH and KCl on division-associated necrosis caused by gene deletions

In order to test how pH stabilization and addition of KCl affected other types of yeast cell death, we also tested its effects on several mutants that exhibit increased rates of cell death during division. These genes were identified in the course of a recent genome-wide screen which used the dye Phloxine B to search for mutants with increased numbers of dead cells in colonies (Alexandrov A.I. et al., manuscript in preparation).

Our data shows that at least 5 of the 12 tested mutants exhibited noticeable reduction of cell death under conditions of stabilized pH 7.0 (Fig. 6A), while addition of KCl to the growth medium had a more universal effect, reducing the death rate of 11 out of the 12 tested mutants, and with effects of larger magnitude (Fig. 6B).

Discussion

Yeasts respond to changes in extracellular pH in a complex manner (Arino 2010; Serra-Cardona et al. 2015) Basically, this response affects the functioning of the main signaling pathways. Until now, there was no data on the effect of extracellular pH on the process of yeast cell death. However, such data have been obtained on metazoan cells. It has been shown that acidification of the extracellular pH changes the manner of cell death of cancer cells from an apoptotic to a necrotic mode (Meurette et al. 2005; Lan et al. 2007) The initial impetus for the present study was a neglect of the dual nature of neutralized ascorbate – which is a scavenger of ROS on one hand, and a pH-buffering agent with pH7.0 on the other. The incubation of *S.cerevisiae* SEY6210 with glucose in 50 mM HEPES buffer pH 7.0 led to a complete suppression of SICD (Fig. 2), which is a case of primary necrosis. Decreasing of the external pH leads to a gradual resumption of SICD. This observation seems to link necrosis with the degree of the extracellular acidification. A similar effect was observed in the induction of apoptosis by valproic acid in the yeast Schizosaccharomyces pombe (Mutoh et al. 2011). We also observed similar mitigating effects of external pH on another, recently discovered mode of death, termed division-associated necrosis (Alexandrov A.I. et al., manuscript in preparation), however death was mitigated only in some of the tested mutations.

As mentioned above, alkalization of the external environment leads to a complex response from the yeast cell. We examined the involvement of some of the signaling pathways and individual genes in the suppression of the necrosis by the neutral pH. In Saccharomyces cerevisiae, the Rim101 pathway senses

external alkalization and alteration in plasma membrane lipid asymmetry through a complex consisted of Rim8, Rim9 and Rim21 at the plasma membrane. It is known that Rim21p, acts as a sensor of extracellular pH (Obara et al. 2012; Nishino et al. 2015). It was logical to assume that deletion of the *rim21* gene could lead to the restoration of SICD in a neutral environment. However, the *Δrim21* strain showed the same suppression of SICD by neutral pH as the parental strain BY4741 (Table 2). We conclude that the Rim101/PacC signaling pathway is not involved in SICD.

The cAMP/protein kinase A (PKA) pathway is one of the major glucose-signaling pathways of budding yeast. Activation of the PKA pathway cause sensitivity to alkaline pH (Casado et al. 2011). Conversely, deactivation of this system increases resistance to alkaline stress. However, activation of the PKA pathway by deleting the *ira2* gene (upstream member of cAMP/PKA pathway) did not affect the development of SICD nor its suppression at neutral pH. Thus, the development of SICD and its suppression by neutral pH turned out to be insensitive to the cAMP level.

It should be noted that the study of the regulation of Rim101 and PKA signaling systems by extracellular pH was carried out at pH 8 (Casado et al. 2011; Obara et al. 2012). We tested the involvement of these systems in the suppression of SICD at pH 7. Perhaps this explains the insensitivity of SICD to the functional state of these signaling systems.

Caspases are a family of protease enzymes playing essential roles in programmed cell death. Inhibition of caspases promote alternative cell death pathways (Vandenabeele et al. 2006). For example, inhibition of caspases induced a switch from apoptosis to necrosis in B lymphocytes (Lemaire et al. 1998). It has also been shown that inhibition of caspases which occurs at acidic pH leads to a change in cell death from apoptosis to necrosis (Lan et al. 2007). Only one metacaspase, Yca1, is present in yeast (Madeo et al. 2002). It is involved in the regulation of apoptosis-like cell death caused by exposure to various treatments such as H_2O_2, acetic acid, salt- and osmotic stress, valproic acid and some metals (Madeo et al. 2009). We reasoned as follows. If metacaspase (by analogy with metazoan caspases), after inhibition in an acidic medium, leads to the switching of apoptosis to necrosis, then in *Δyca1* strain, we either should not observe SICD in an acidic medium, or SICD will also be observed at neutral pH. However, the Δ*yca1* mutant did not show any of the expected responses (Table 2). Therefore, *yca1* is not involved in SICD. This is in agreement with published data that only about half of the cell death scenarios are caspase-dependent (Madeo et al. 2009).

As noted earlier, SICD occurs when the ROS content is high (Valiakhmetov et al. 2019). Two major sources of ROS are known in the yeast cell - the mitochondrial respiratory chain and NADPH oxidase (Rinnerthaler et al. 2012) of the ER. We found no data on regulation of ROS production by the ambient pH. However, if such regulation existed, it would explain the fact that SICD was suppressed by neutral pH. To test this assumption, we used two deletion mutants: Δ*afo1*(Heeren et al. 2009) with reduced mitochondrial ROS generation due to the lack of a large subunit of the mitochondrial ribosome and Δ*yno1* in which there is no NADPH oxidase (Rinnerthaler et al. 2012). Surprisingly, both strains continued to exhibit generation of ROS when cells were incubated with glucose in an unbuffered medium. The

percentage of cells with ROS (and with SICD) roughly coincided with the percentage of such cells in the parental strain BY4741 (Table 2.). And just as the parent strain, both strains showed a pH dependence of the SICD. Incubation with glucose at pH 7 resulted in the suppression of the number of cells with ROS (and with SICD) by 90%. The data obtained indicate that the generation of ROS by the respiratory chain of mitochondria or NADPH oxidase of ER is not regulated by the extracellular pH.

However, the level of ROS in the cell can be indirectly altered by actin dynamics. It was previously shown that a decrease in actin dynamics leads to depolarization of the mitochondrial membrane and an increase in ROS generation (Gourlay et al. 2004; Gourlay and Ayscough 2005a). To test the involvement of genes regulating actin dynamics, we used three strains: Δ*end3*, Δ*scp1* and Δ*sla1*. Scp1p is the small actin-binding protein related to mammalian SM22/transgelin (Goodman et al. 2003; Winder et al. 2003). Sla1p is the endocytic adaptor protein that interacts with ubiquitin and involved in endocytosis and actin cortical patch assembly (Warren et al. 2002). End3p is the EH domain-containing protein involved in endocytosis, actin cytoskeletal organization and cell wall morphogenesis and forms a complex with Sla1p (Tang et al. 1997). In wild-type cells, Scp1p decreases actin dynamics and thus increases the amount of cellular ROS. The *Δscp1* cells, respectively, have a reduced amount of ROS. Conversely, in wild cells, Sla1p and End3p increase actin dynamics, which leads to a decrease in the amount of ROS. In strains *Δsla1* and *Δend3*, respectively, the ROS level is increased. Incubation of the Δ*end3*, Δ*scp1* and Δ*sla1*strains with glucose in water showed that cells with deleted genes generate ROS and undergo SICD at approximately the same efficiency as the parent strain. Incubation of these strains with glucose at pH 7.0 resulted in a complete absence of ROS and SICD (Table 2). Such results are to be expected, since End3p, Scp1p and Sla1p alter the ROS level indirectly by modulating the membrane potential of mitochondria, which, as can be seen from experiments with Δafo1, are not involved in the development of SICD.

Since we did not observe the sensitivity of SICD to neutral pH to depend on the functioning of several tested cellular systems, we hypothesized that $\Delta\mu H^+$ might be involved in this effect. During incubation with glucose, *S.cerevisiae* cells decreases the pH of the medium to pH 3.7 after 5 minutes (Fig. 1). This creates a large ΔpH on the plasma membrane, which is absent when cells are incubated at 50 mM HEPES pH 7. Hence dissipation of ΔpH by the protonophore should lead to the suppression of SICD. Figure 3 shows that DNP suppresses SICD by more than 80% already at a concentration of 0.5 mM. In this case, the extracellular pH was 4.7 (Fig. 1), which is significantly higher than the value for glucose metabolism in the absence of DNP (pH 3.5). It is noteworthy that the ROS level also dropped by more than 80%. The role of the second component of the membrane potential - ΔΨ - was verified in experiments with exogenous KCl. The ROS generation and SICD was suppressed by 80% when *S.cerevisiae* SEY6210 is incubated with glucose in the presence of 150 mM KCl (Fig. 4). Interestingly, the same data were obtained in experiments with yeast in the stationary growth phase, though at lower KCl concentrations (Hoeberichts et al. 2010). In order to make sure that the exogenous KCl really affected ΔΨ rather than non-specifically inhibits SICD we used DiOC2(3) dye to monitor changes in plasma membrane potential (Fig. 5). Since the dye enters cells in a voltage-dependent manner, it was important to exclude

the contribution of the mitochondrial membrane potential to the observed changes in ΔΨ. Strain SEY6210 rho^0 showed the same SICD development as the wild type (Table 2). Therefore, we used this strain to record changes in ΔΨ on the plasma membrane. As can be seen from Fig. 5, the incubation of cells in an aqueous solution of glucose leads to a significant increase in ΔΨ. Incubation of cells with glucose in a buffer with pH7.0 or in the presence of 150 mM KCl completely suppresses this increase of ΔΨ. It is important to note that the acidification of the medium in the presence of 150 mM KCl even slightly exceeded acidification during incubation with glucose only (Fig. 1). It is known that extracellular K^+ is a regulator of the membrane potential on the plasma membrane of yeast(Madrid et al. 1998) and causes additional acidification of the medium (Martinez-Munoz and Kane 2008). An 80% inhibition of the ROS generation and SICD development under these conditions suggests that ΔΨ, rather than ΔpH, is responsible for ROS generation and subsequent development of SICD. Whether a hyperpolarized membrane can generate ROS on its own, or some abnormal cellular process is triggered via this hyperpolarization, is currently unclear. However, since we found a direct correlation between the ROS generation and the magnitude of ΔΨ on the plasma membrane, we do not exclude the possibility of ROS production by some membrane-bound complexes at a high ΔΨ value. This is supported by the fact that the removal of any of the two main producers of ROS - Afo1p or Yno1p - had no effect on ROS generation in our conditions. It should also be emphasized that an important issue is the selection of a suitable buffer to study the effect of extracellular pH on the development of primary necrosis. The presence of high concentrations of inorganic ions in buffers with pH 3.5, such as K^+, Na^+, citrate, phthalate and tartrate, distorts the real picture, because monovalent ions significantly change $\Delta\mu H^+$. Therefore, to test the SICD at an initially low pH of 3.5, we used water acidified with HCl. Notably, dissipating the ΔΨ on the plasma membrane also had profound effects on nearly all of the tested mutations causing rapid division-associated necrosis.

Summarizing the obtained data, we can state that both SICD and rapid division associated necrosis in some mutants are suppressed 1) upon dissipation of ΔpH at neutral pH (or treatment with DNP for SICD) and 2) upon dissipation of ΔΨ in the presence of 150 mM extracellular KCl. Thus, our current understanding of SICD, as well as, possibly, a subset of necrotic death associated with some mutations, is summarized in Fig. 7. ATP, synthesized during glucose metabolism, is consumed by the plasma membrane H-ATPase to create and maintain membrane potential through the extrusion of protons into the extracellular environment. When yeast grows on a rich medium, the transport of K^+ into the cell prevents membrane hyperpolarization. The conditions of SICD (i.e. incubation in deionized water in the presence of glucose) do not provide sufficient ions to counterbalance the hyperpolarization, thus triggering some yet uncharacterized process resulting in necrosis. This notion is supported by the recent observations that deletion of K^+ transporters of the Trk-family results in exacerbated SICD (Dušková et al. 2021), and coincides with the previously stated hypothesis about the involvement of the membrane potential in SICD observed in cells in the stationary growth phase (Hoeberichts et al. 2010). Notably, addition of KCl and stabilization of alkaline pH have been reported to be highly efficient methods of increasing yeast tolerance to ethanol, which also perturbs membrane structure and may thus cause necrotic cell death (Lam et al. 2014). Overall, our observations and the data in the literature suggest that

deciphering the interplay between membrane potential and cell death may have wide-ranging ramifications for the understanding of cell death mechanisms in yeast.

Declarations

Conflicts of interest

The authors declare that they have no competing interests.

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Figures

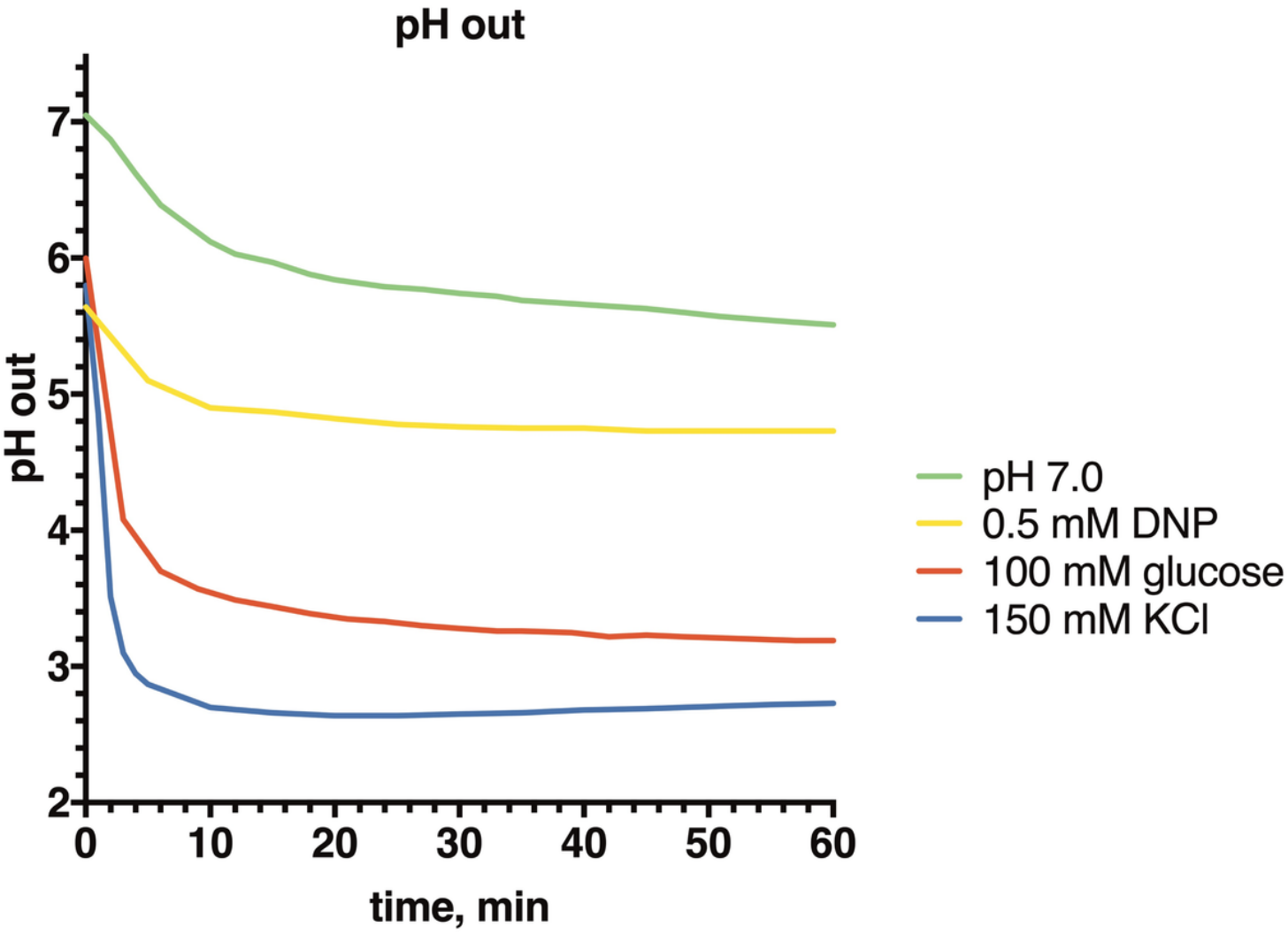

Figure 1

Acidification of the external medium by yeast SEY6210 during incubation with 100 mM glucose under various conditions. pH7.0 – incubation in 50 mM HEPES buffer pH 7.0; 0.5 mM DNP – incubation in nonbuffered media in the presence of 0.5 mM dinitrophenol (DNP); 100 mM glucose – incubation in nonbuffered media without any additions; 150 mM KCl – incubation in nonbuffered media supplemented with 150 mM KCl

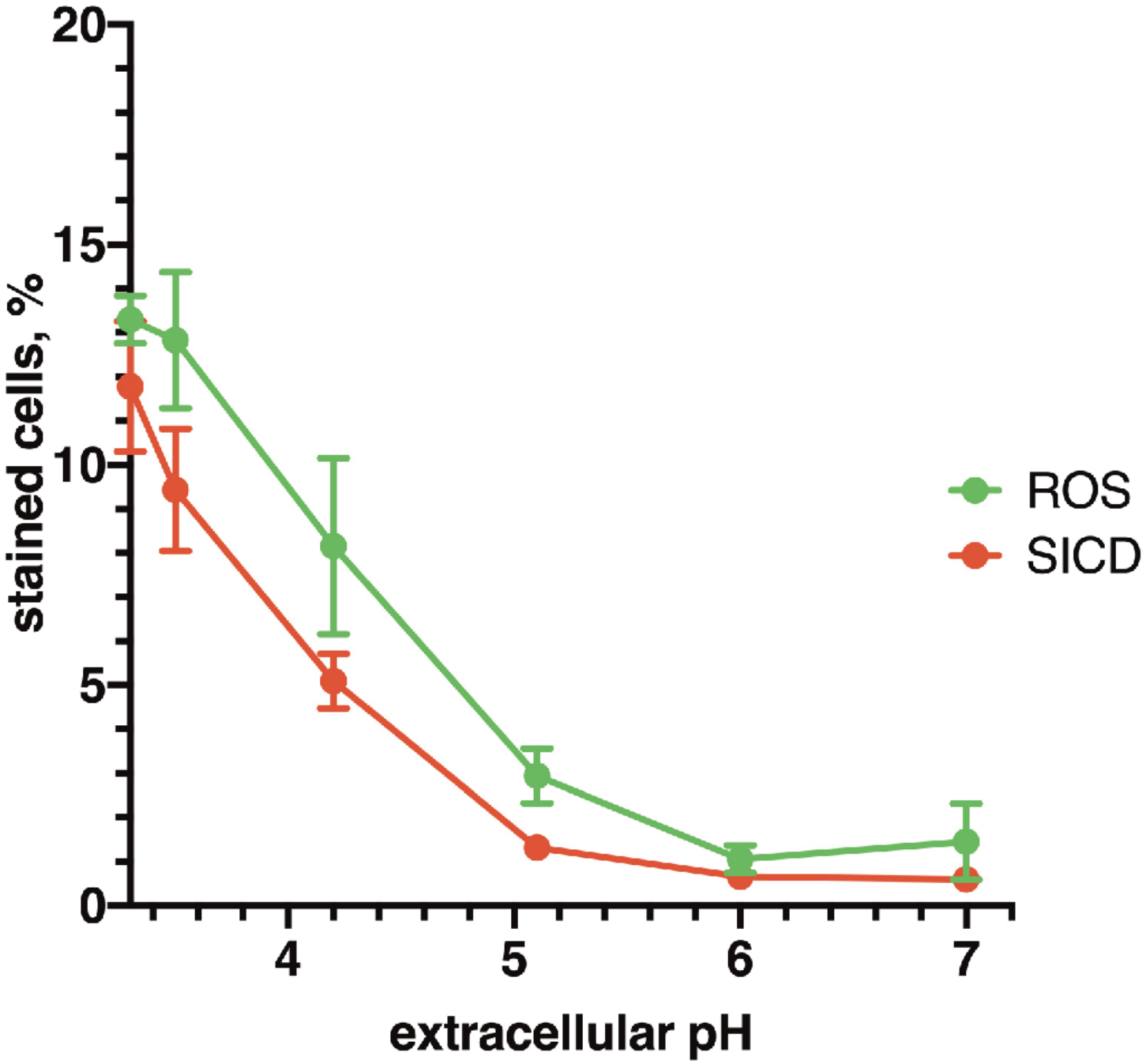

Figure 2

Dependence of ROS generation and SICD development on extracellular pH (Mean±SD). ROS – percentage of the cells with ROS as estimated by DHR staining; SICD – percentage of the cells with SICD as

estimated by FD staining. Buffer used for pH 7 – 50 mM HEPES; pH 6 and 5.1 - 20 mM MES; pH 4.2 – 20 mM glycine-glycine; pH 3.5 and starting point pH 3.3 – water acidified by HCl

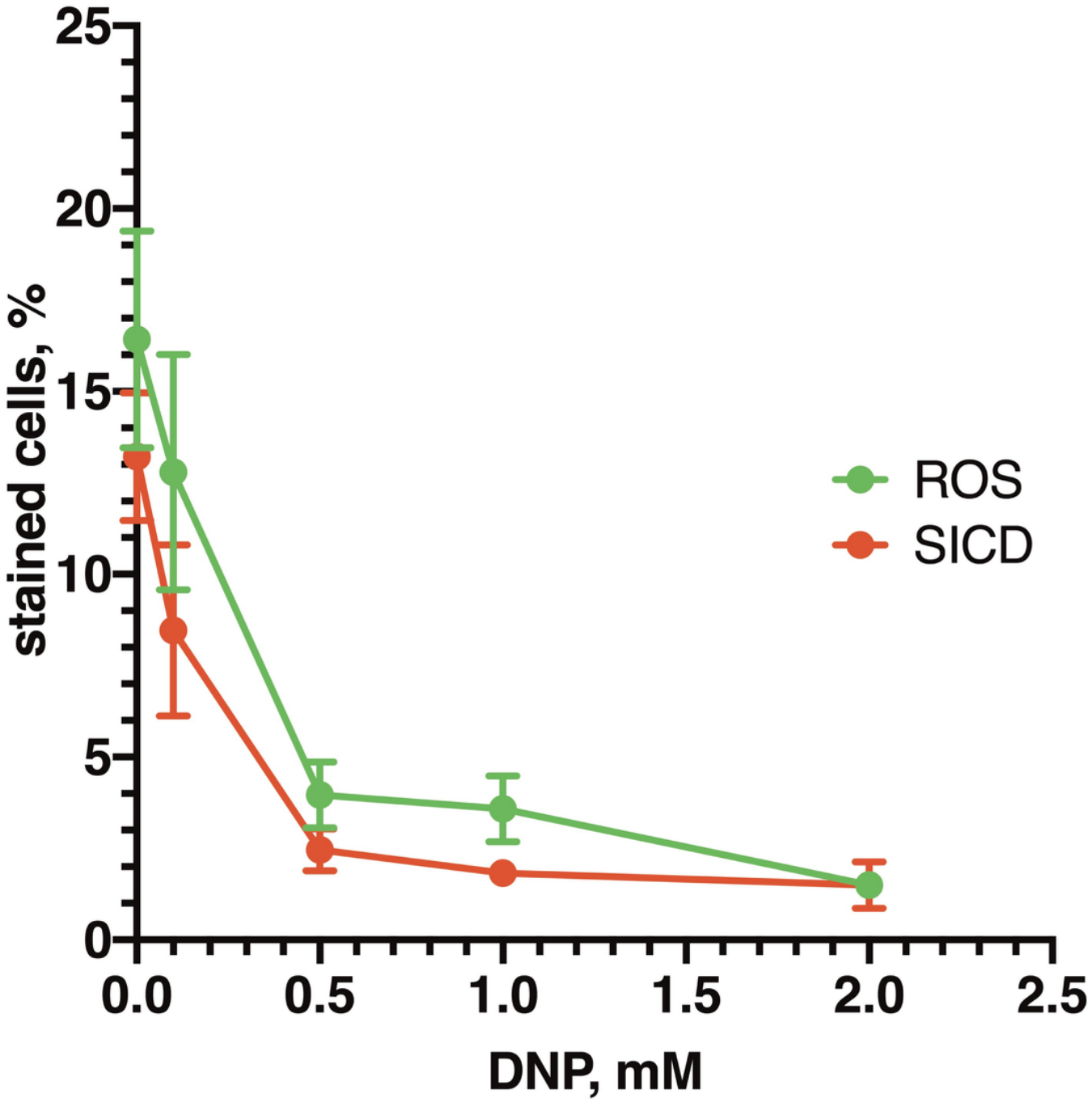

Figure 3

Effect of DNP on ROS generation and SICD development in SEY6210 strain (Mean±SD). Cells were prepared as in Materials and Methods section. DNP was added simultaneously with glucose. In 1 h percentage of the cells with ROS and SICD was estimated by flow cytometry. Cells with ROS were determined by the DHR staining and cells with SICD were determined by the FD staining

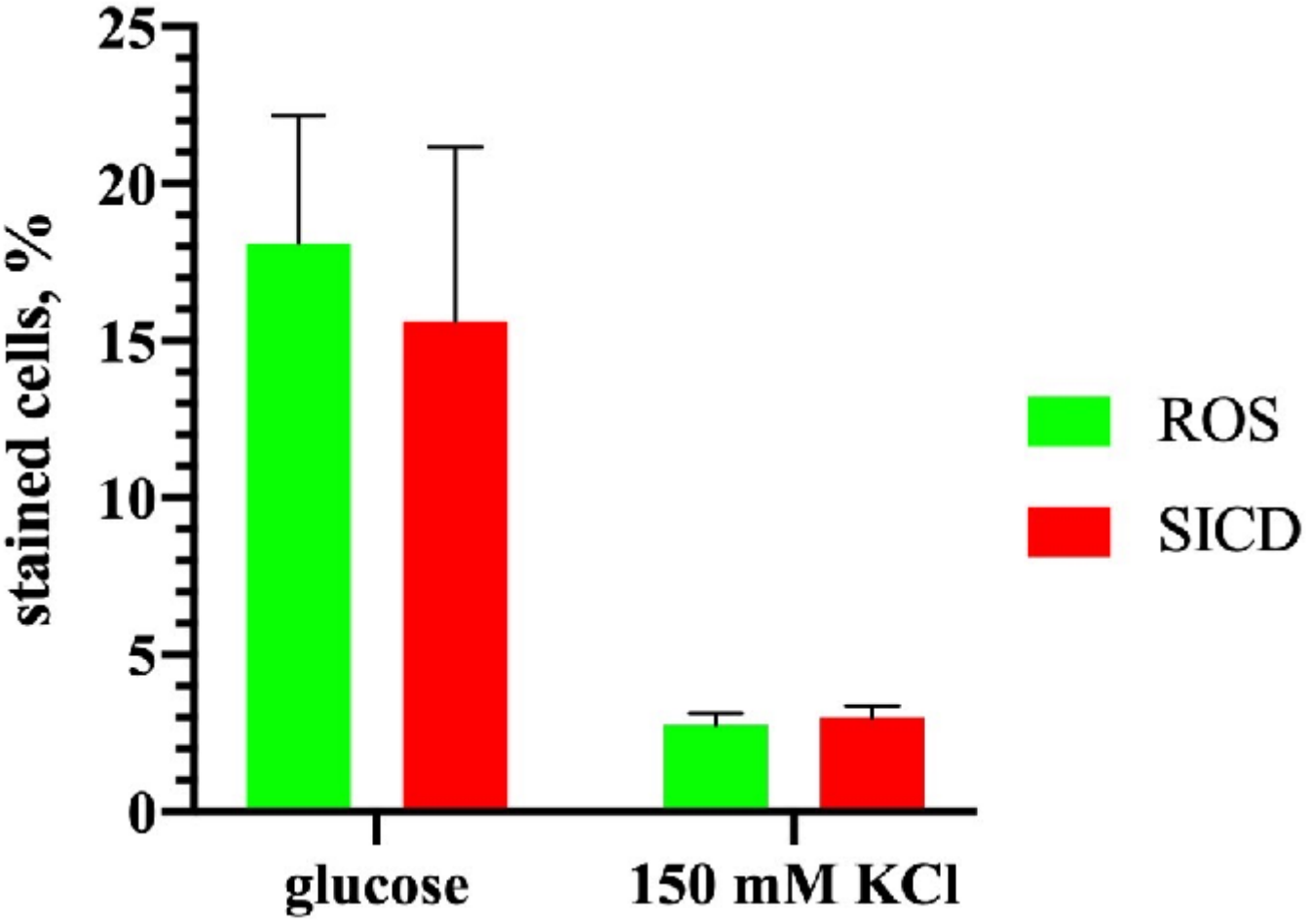

Figure 4

Effect of addition of 150 mM KCl in incubation medium on ROS generation and SICD development in cells of SEY6210 strain (Mean±SD). Cells with ROS were determined by the DHR staining and cells with SICD were determined by the FD staining

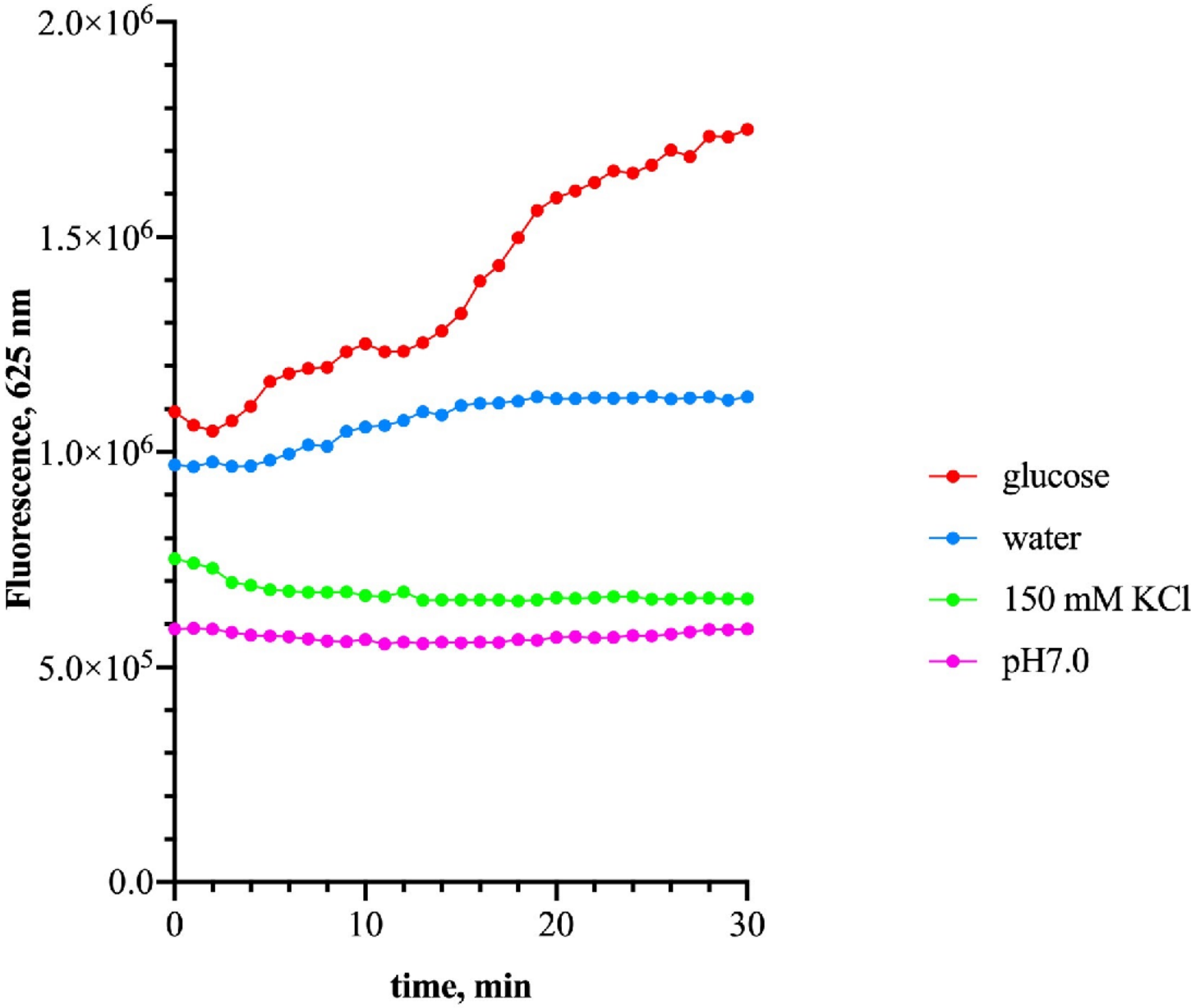

Figure 5

Dynamics of the membrane potential – ΔΨ – on the plasma membrane of SEY6210 rho0 strain. ΔΨ dynamics was tracked by the change of the fluorescence of DiOC2(3) dye. Cells were prepared as described under Culture growth section. 0.05 mL of cells were added to 1 mL of MilliQ water or 1 mL of 50 mM HEPES pH 7.0. 0.1 mL of this cell suspension was plated in a 96-well black plate. 1 µL of 3 mM DiOC2 (3) in DMSO was added to give a final dye concentration of 30 µM. The plate was incubated for 5 min at 30 ° C to distribute the dye. To initiate the process, 5 µL of 2M glucose was added to the wells. Fluorescence was recorded on a FilterMax F5 plate reader. Ex 485 nm/Em 625 nm. Readings were taken every minute with 30 second shakings before each measurement. Glucose – incubation of the cells with 100 mM glucose in nonbuffered medium. Water – incubation of the cells in water without glucose. 150 mM KCl – incubation of the cells with 100 mM glucose in the presence 150 mM KCl. pH7.0 – incubation of the cells with 100 mM glucose in 50 mM HEPES buffer

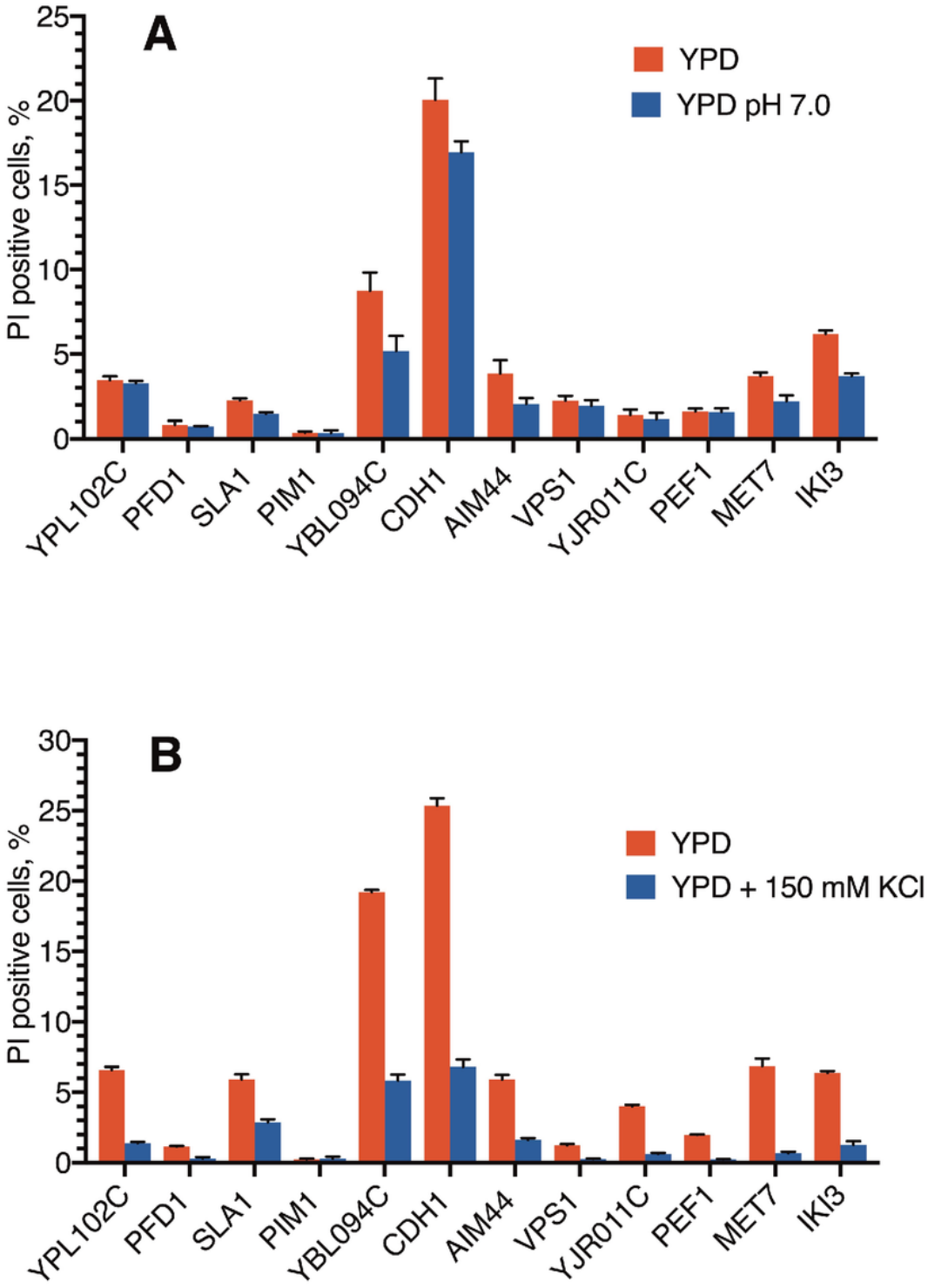

Figure 6

Stabilization of external pH and addition of KCl mitigates cell death in mutants with increased rates of cell death (Mean±SD). Cells of the BY4741 strains harboring the noted mutations were cultivated in YPD or YPD supplemented with buffering agent (A) or KCl (150mM) (B) and cultivated for 19 hours. The final optical density of all strains was below 0.3, i.e., they were in the logarithmic growth phase. The percentage of dead cells was determined by PI staining

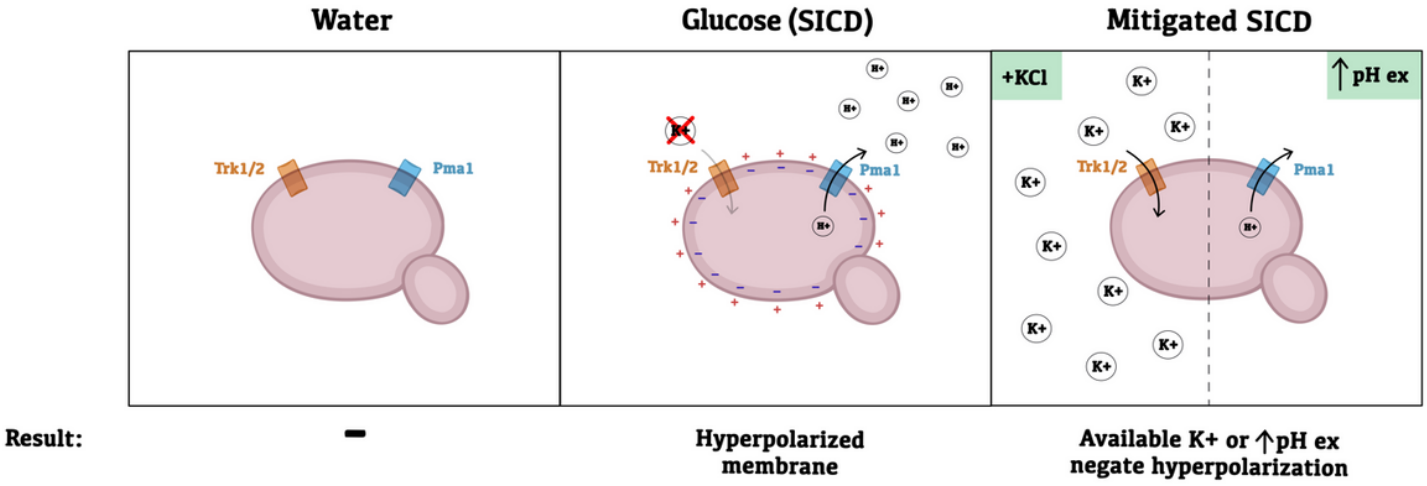

Figure 7

Proposed mechanism of membrane hyperpolarization during SICD and its mitigation. See text for details