Research Article

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Elucidating the effects of restrictive temperatures on the function of Arl1p in ion homeostasis in *Saccharomyces cerevisiae*

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Abstract

Strict regulation of ion homeostasis is necessary for cell function and survival. Arl1p, a member of the ADP-ribosylation factor-like protein family, is known to function in ion homeostasis by mediating lithium (Li^+) tolerance and regulating potassium ion (K^+) influx in *Saccharomyces cerevisiae.* Given that *arl1Δ* mutants show temperature sensitivity, this study investigates the effects of restrictive temperatures on the function of Arl1p in regulating Li^+ tolerance and the role of K^+ in enhancing Li^+ tolerance. We have characterized cell growth rates of *arl1Δ* mutant and wild-type cells in the presence of Li^+ and K^+ at optimal (30°C) and restrictive (37°C) temperatures using haemocytometry. We report that Li^+ sensitivity was exacerbated at restrictive temperatures in the wild-type cells, but not in *arl1Δ* mutants, suggesting the potential temperature sensitivity of Arl1p. Further, we report that K^+ was sufficient to suppress Li^+-induced decreases in cell growth in wild-type and *arl1Δ* mutant cells at optimal and restrictive temperatures, suggesting that K^+ functions as a growth factor in *S. cerevisiae.* Future studies may aim to further elucidate the relationship between temperature and ion homeostasis.

Introduction

Arl1p forms part of a highly conserved group of ADP-ribosylation-like (Arl) proteins that mediate functions in the trans-Golgi Network (TGN), such as vesicle trafficking, membrane remodeling, and maintenance of ion homeostasis (Yu & Lee, 2017). *arl1Δ* mutants, however, have been observed to disrupt the regulatory functions, specifically when involved in parallel pathways and mechanisms which resemble transduction pathways that include autophagy and ion homeostasis.

arl1Δ mutants demonstrate sensitivity to lithium ions (Li^+), which was concluded by an observed reduction in cell growth when exposed to Li^+ during growth (Munson et al., 2004b). Increased sensitivity to the ion was observed in double loss of function mutants of *ARL1* and a secondary protein, suggesting that there is a parallel pathway present where Arl1p functions with other proteins to mediate Li^+ tolerance

(Munson et al., 2004b). Further, Li^+ sensitivity was suppressed by the addition of potassium ions (K^+) to the media, showing restored cell growth (Munson et al., 2004b). The suppressed sensitivity to Li^+ through the addition of K^+ is a result of Arl1p's role in the regulation of K^+ translocation proteins Trk1 and Trk2 through the stimulation of calcineurin, a protein which controls lithium influx and efflux (Munson et al., 2004a).

The regulatory role of Arl1p on Trk1p and Trk2p was determined as *arl1Δ* mutants showed decreased K^+ influx and membrane hyperpolarization, but no change in efflux (Munson et al., 2004a). K^+ is reported to be involved in cell growth through osmosis (Borovikova et al., 2013). This is due to the ability of K^+ to bind to water molecules entering the cell, thus increasing the factors that influence cell growth and division, such as cell size and turgor pressure.

Disturbances in Li^+ and K^+ homeostasis can cause lethal effects on the cell or initiate mechanisms for cell survival such as autophagy. Autophagy is a process of consumption or recycling of proteins and internal contents that are no longer useful for the cell (Das et al., 2012). Li^+ and K^+ both are involved in autophagy; excess Li^+ and K^+ starvation both induce autophagy (Sakar et al., 2005; Rangarajan, 2020).

arl1Δ mutants experience impaired autophagy at restrictive temperatures, but the same is not observed when exposed to optimal temperatures due to parallel functions of Arl1p with another protein, Ypt6p (Yang & Rosenwald, 2016). At optimal temperatures, even in *arl1Δ* mutants, Ypt6p performs the functions required to regulate the formation of autophagosomes (Yang & Rosenwald, 2016). However, at restrictive temperatures the function of Ypt6p is affected, leading to impaired autophagy pathways.

Although prior studies elucidate the function of Arl1p in ion homeostasis and suggest that *arl1Δ* mutants are temperature sensitive, it is unknown whether temperature affects the function of Arl1p in maintaining ion homeostasis. Hence, our study aims to determine whether restrictive temperature heightens Li^+ sensitivity in *arl1Δ* mutants and whether K^+ remains sufficient to suppress Li^+ sensitivity in *arl1Δ* mutants. We hypothesized that under restrictive temperatures, *arl1Δ* mutants will exhibit increased sensitivity to Li^+, demonstrated by reduced cell growth and that K^+ will remain sufficient to suppress Li^+ sensitivity in *arl1Δ* mutants, demonstrated by restored cell growth.

Since *arl1Δ* mutants possess impaired regulation of Trk1p and Trk2p due to the lack of Arl1p, we expect that cells exposed to higher temperatures would not affect these translocation proteins, and rather, show reduced cell growths due to toxic cation presence and heat shock response. Previous studies have observed a change in carbohydrate flux when cells were exposed to restrictive temperature conditions (Morano et al., 2012). With an alteration in glycolysis under stress causing less nutrient uptake and impaired autophagy, cell growth in *arl1Δ* mutants would likely be lower than those of the wild-type strain and mutants cultured at permissive temperatures. Additionally, the presence of Li^+ has been found to increase the porosity of the cell wall, making it more permeable (Chen et al., 2008). This allows Li^+ to enter the cell even when translocation proteins are impaired.

To determine the effects of temperature on cation sensitivity in *arl1Δ* mutants, we treated cells with liquid yeast/peptone/dextrose (YPD) media containing lithium chloride (LiCl) and YPD media containing both potassium chloride (KCl) and LiCl at optimal and restrictive temperatures. After incubation, haemocytometer counts of each media were conducted to determine cell growth correspondingly. We report that Li^+ sensitivity was exacerbated at restrictive temperatures in the wild-type cells, but not in *arl1Δ* mutants, suggesting the potential temperature sensitivity of Arl1p. Further, we report that K^+ suppressed Li^+-induced decreases in cell growth in wild-type and *arl1Δ* mutant cells at optimal and restrictive temperatures, suggesting that K^+ acts as a growth factor in *S. cerevisiae.*

Methods

All materials and equipment were provided by the BIOL 340 laboratory (Moussavi 2023). Visual overviews of experiments can be found in the appendix.

Cell culture

The liquid YPD media used in all cell cultures in both experiments consisted of 10 g/L of yeast extract, 20 g/L of peptone, and 20 g/L of dextrose (Moussavi, 2023). Liquid YPD media with LiCl contained 75 mM of LiCl, media with KCl contained 250 mM of KCl, and media with both LiCl and KCl were 75 mM and 250 mM, respectively, as demonstrated in Munson et. al. (2004b). Each strain of *S. cerevisiae* was incubated in each type of media for 24 hours in shakers set to 30°C and 37°C.

Haemocytometry

BY4741a (MATa; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0) wildtype (SGD, www.yeastgenome.org) and the *arl1Δ* mutant strain cell concentrations were quantified using haemocytometry and light microscopes at 10X objective. Cell cultures were diluted with YPD before counting, such that each of the cell counts ranged from 30-100 cells. Five technical replicates were recorded per sample condition, where at least three of the replicates lie within two standard deviations of each other. The protocol for measuring cell concentration using haemocytometry is found in the BIOL 340 Lab Manual (Moussavi, 2023).

Data and statistical analyses

Cell count fold changes were calculated using the following formula: (Y-X)/X. Positive values indicate an increase and negative values indicate a decrease. One-way ANOVA test was conducted using GraphPad Prism version 9.0.0 for Windows, GraphPad Software, San Diego, California USA, www.graphpad.com. A one-way ANOVA method was used between all genotypes and treatments to compare the differences between the mean cell counts. Error bars represent standard deviation. The symbols *, **, ***, and **** in Figure 1 and Figure 2 represent p-values of < 0.05, <0.01, <0.001, and <0.0001 respectively.

Results

Restrictive temperatures did not exacerbate Li^+ sensitivity in *arl1Δ* mutants

To determine the cell growth of wild-type and *arl1Δ* mutants in optimal conditions, we first treated the cells with YPD media at 30°C. Surprisingly, cell concentration was significantly higher ($p < 0.0001$) for the *arl1Δ* mutants than the wild-type cells cultured in YPD at 30°C by 0.69-fold, suggesting greater growth of *arl1Δ* mutants in optimal conditions compared to wild-type cells (Figure 1A). Further, to confirm that *arl1Δ* mutants are temperature-sensitive, we treated *arlΔ* mutant cells with YPD at restrictive temperatures (37°C) (Yang & Rosenwald, 2016). We found that there was a significant decrease ($p < 0.0001$) in *arlΔ* mutant cell concentrations in restrictive temperatures when compared to optimal temperatures by -0.44-fold (Figure 1A).

To determine whether restrictive temperatures would enhance Li^+ sensitivity in *arl1Δ* mutants, we obtained the cell concentration of wild-type and *arl1Δ* mutant cells treated with YPD media containing LiCl in optimal (30°C) and restrictive (37°C) temperatures (Figure 1). In LiCl-containing YPD media at both optimal and restrictive temperatures, wild-type and *arl1Δ* mutant cells showed decreased cell concentrations, relative to cells cultured in YPD media, suggesting that wild-type and *arl1Δ* mutant cells are both sensitive to Li^+ (Figure 1A). Despite this, the decrease in cell concentration in LiCl-containing YPD media was greater for *arl1Δ* mutant cells than for wild-type cells in optimal temperatures, suggesting heightened Li^+ sensitivity in *arl1Δ* mutants.

There was no significant difference between *arl1Δ* mutant cells grown in LiCl-containing media cultured at 30°C and 37°C conditions (Figure 1B and 1C). Together, the results suggest that while restrictive temperatures impair the cell growth of *arl1Δ* mutants, there is no enhancement in LiCl sensitivity in *arl1Δ* mutants at restrictive temperatures. Cell concentration was significantly lower in wild-type cells cultured in LiCl-containing media at 37°C than at 30°C by a -0.58-fold change. This suggests that LiCl sensitivity was increased in restrictive temperatures for wild-type cells (Figure 1C).

Provided that we observed high cell concentration for *arl1Δ* mutant cultured in KCl-containing, we can conclude that Li^+, rather than Cl^- is likely responsible for the decrease in growth observed for both wild-

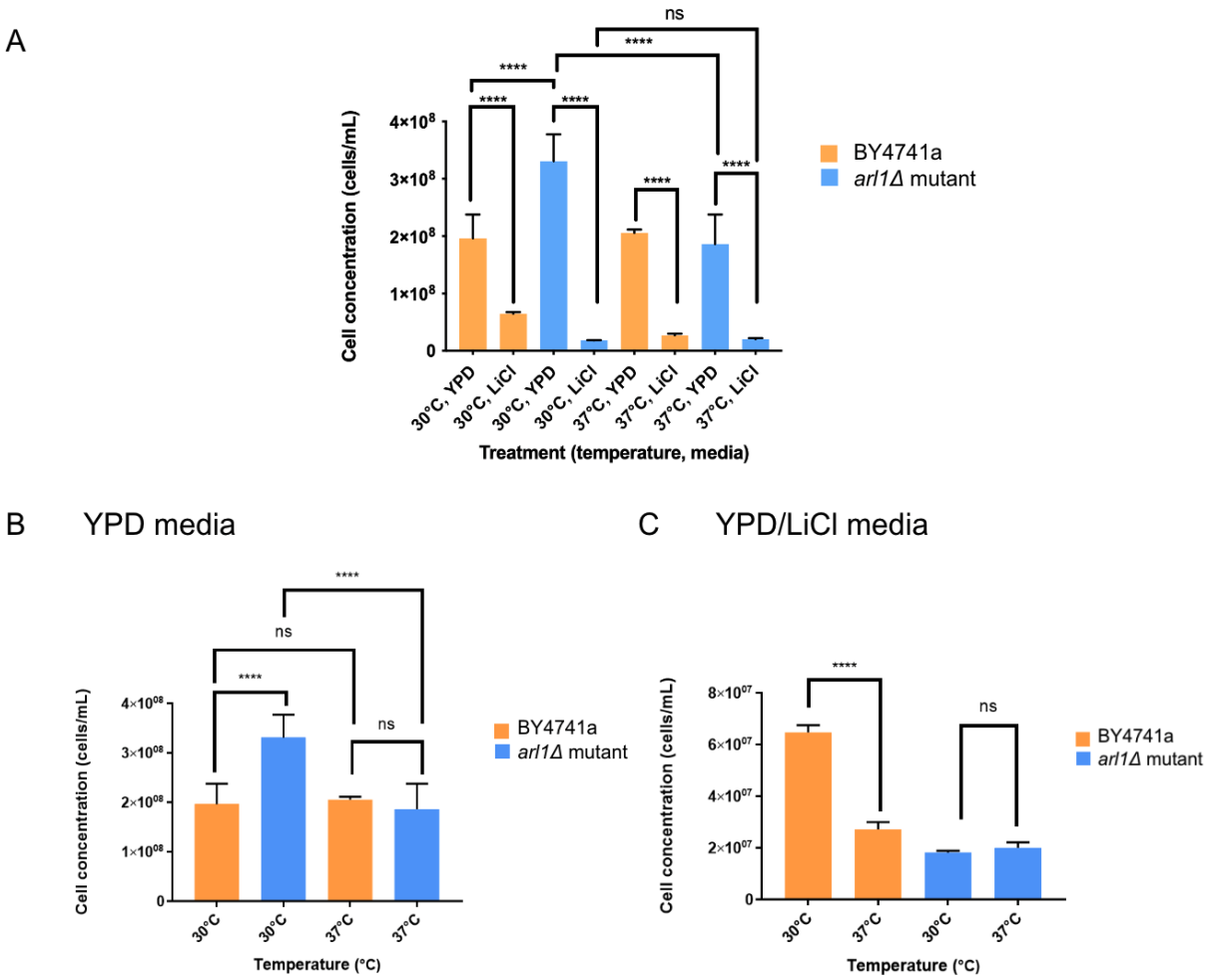

Figure 1. Cell concentrations of BY4741a and *arlΔ* mutant cells in LiCl-containing YPD. (A-C) Error bars indicate standard deviation. *n = 5*. ns: not significant; ****: p < 0.0001. **(A)** Cell concentration in BY4741a and *arlΔ* mutant cells in YPD and LiCl-containing YPD media at optimal and restrictive temperatures. **(B)** Cell concentrations of BY4741a and *arlΔ* mutant cells in YPD. **(C)** Cell concentrations of BY4741a and *arlΔ* mutant cells in LiCl-containing YPD media.

type and *arl1Δ* mutant cells (data shown in Figure 2).

K^+ remains sufficient to suppress Li^+ sensitivity in *arl1Δ* mutants at restrictive temperatures

To determine whether KCl remained sufficient to suppress Li^+ sensitivity in *arl1Δ* mutants at restrictive temperatures, we quantified the cell concentration of wild-type and *arl1Δ* mutants in optimal (30°C) and restrictive temperatures (37°C) in YPD containing both LiCl and KCl (Figure 2). Given that *arl1Δ* mutants did not show exacerbated Li^+ sensitivity at restrictive temperatures, we predicted that KCl addition to the media would continue to suppress the growth defects observed in *arl1Δ* mutants treated with LiCl-containing YPD media. In the wild-type strain, cell concentration increased in the KCl and LiCl-containing YPD media, relative to the LiCl-containing media at optimal and restrictive temperatures by 1.62-fold and 2.76-fold, respectively (Figure 2A). In the *arl1Δ* mutant strains, cell concentration increased in the KCl and LiCl-containing YPD media, relative to the LiCl-containing media at optimal and restrictive temperatures by 12.12-fold and 8.45-fold, respectively (Figure 2A). Together, the results suggest that K^+ is sufficient to suppress Li^+ sensitivity in wild-type and mutant strains and that temperature does not affect this suppression. The cell concentration of wild-type cells treated with KCl-containing YPD media at optimal temperatures was significantly higher than the control group where wild-type cells were treated with YPD media at optimal temperatures by a 0.36-fold change, further

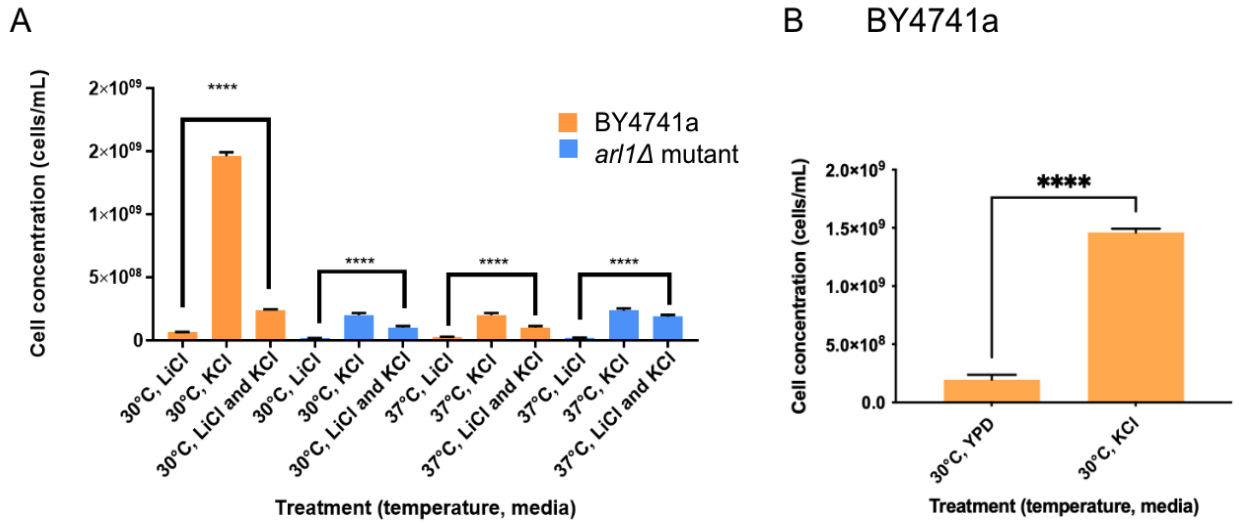

Figure 2. Cell concentrations of BY4741a and *arl1Δ* mutant cells in LiCl and KCl-containing YPD media. (A-B) Error bars indicate standard deviation. $n = 5$. ****: $p < 0.0001$. **(A)** Cell concentrations of BY4741a (wild-type) and *arl1Δ* mutant cells in LiCl, KCl-, and LiCl/KCl-containing YPD media. **(B)** Cell concentrations of BY4741a cells in optimal temperatures in YPD and KCl-containing YPD media.

suggesting the role of K^+ in promoting cell growth (Figure 2B).

Discussion

In this study, we have investigated the effects of restrictive temperatures on Li^+ tolerance in *arl1Δ* mutants and whether K^+ remains sufficient to suppress the Li^+-induced inhibition of cell growth in *arl1Δ* mutants at restrictive temperatures (Munson et al., 2004b). Our study shows that Li^+ sensitivity is exacerbated at restrictive temperatures in wild-type cells, but not in *arl1Δ* mutant cells. Further, our study shows that K^+ promotes cell growth in Li^+-treated wild-type and *arl1Δ* mutant cells, suggesting the role of K^+ as a growth factor.

The results of our study confirmed the temperature sensitivity of wild-type and *arl1Δ* mutants that were previously reported by Marešová et al. (2012). Our results showed a decrease in cell concentration in both wild-type and *arl1Δ* mutants between optimal (30°C) and restrictive (37°C) temperatures when grown in YPD media (Figure 1B).

arl1Δ mutants exhibit higher cell growth than wild-type cells in optimal conditions

Prior studies have reported that while *arl1Δ* mutant cells exhibit temperature sensitivity in yeast cells, they do not exhibit synthetic lethality (Rosenwald et al., 2002; Marešová et al., 2012). The results of our study confirmed the temperature sensitivity of *arl1Δ* mutants and found that wild-type cells exhibit a similar temperature sensitivity. In both wild-type and *arl1Δ* mutants, cell concentrations decreased when cells were treated with restrictive (37°C) temperatures in YPD media (Figure 1B).

Contrary to prior findings by Rosenwald et al. (2002), where *arl1Δ* mutant cells were reported to have synonymous cell growth to wild-type cells, we found that *arl1Δ* mutant cells had a higher cell concentration than wild-type cells when cultured at permissive temperatures (30°C). Given that Arl1p is known to play a role in autophagy and high autophagy rates are known to be associated with lower cell growth, it is possible that *arl1Δ* mutants had higher cell growth in optimal conditions than wild-type cells because an impaired autophagy pathway may lead to greater cell growth (Wang & Levine, 2010).

Restrictive temperatures exacerbate Li^+ sensitivity in wild-type cells

Lithium induces autophagy by inhibiting enzymes that limit IMPase inhibitory activity, resulting in decreased cell growth (Sarkar et al., 2005). Given that *arl1Δ* mutants were found to be temperature-sensitive, we aimed to investigate whether restrictive

temperatures would enhance Li^+-induced reduction in cell growth. Contrary to our predictions, our findings show that restrictive temperatures enhance Li^+ sensitivity in wild-type cells, but not in *arl1Δ* mutants. The enhanced Li^+ sensitivity of the wild-type strain may be explained by the combined effects of Li^+ treatment and heat stress on cell permeability. Chen et al. (2008) reported that Li^+ treatments result in modifications in the porosity of the cell wall, leading to increased cell permeability and thus increased ion sensitivity. Furthermore, heat-induced stress may change cell membrane integrity and fluidity (Guyot et al., 2015). This leads to increased membrane permeability which further translates to increased ion sensitivity. The combination of these two factors could explain the increased Li^+ sensitivity found in wild-type cells at restrictive temperatures.

Given that the Li^+ sensitivity in wild-type cells treated with restrictive temperatures resulted in cell concentrations that were similar to those of the *arl1Δ* mutants, heat stress may impair the biological functions of Arl1p. This may explain why we did not observe exacerbated Li^+ sensitivity in *arlΔ* mutants at restrictive temperatures - if Arl1p is not present in the cell, there would be no proteins whose function would be impaired by temperature. Furthermore, it is possible that additional stressors, such as heat do not further exacerbate Li^+ sensitivity in a*rl1Δ* mutants due to other compensatory mechanisms that allow cells to withstand multiple stressors, such that the cellular process that is affected by Arl1p are already fully compromised and cannot be further impaired by additional variables. The potential temperature sensitivity of Arl1p may be explored in future studies.

K^+ suppresses Li^+ sensitivity in both wild-type and *arl1Δ* mutant cells

The findings of our study support the literature that K^+ is sufficient to suppress Li^+-induced cell growth defects in both wild-type and *arl1Δ* mutant cells at optimal temperatures and further elucidates that this mechanism remains intact at restrictive temperatures (Munson et al., 2004b). Furthermore, we find that the addition of KCl to YPD media leads to a significant increase in cell concentrations for both wild-type and mutant strains, suggesting the role of K^+ as a growth factor. Although *arl1* loss of function negatively affects the cation homeostasis of *S. cerevisiae,* augmentation of calcineurin activity may result from the addition of KCl. Calcineurin plays a role in K^+ transporter regulation and participates in reducing Li^+ influx and increasing Li^+ efflux by increasing TRK1, TRK2 and ENA1 expression (Munson et al., 2004b). As a result, Li^+ sensitivity may be obscured, resulting in decreased autophagy and increased cell concentration. The findings suggest that while *arl1* is required for K^+ influx, alternative K^+ influx pathways must be sufficient to maintain ion homeostasis (Munson et al., 2004a). Future studies may explore the molecular identities of the parallel K^+ influx/efflux pathways that regulate K^+ homeostasis in cells.

Conclusion

The precise regulation of ion homeostasis is necessary for cell function and survival. In conclusion, our study reports that restrictive temperatures affect the ion sensitivity of wild-type *S. cerevisiae*, suggesting a relationship between temperature and the regulation of ion homeostasis. Given that ion sensitivity was not exacerbated in *arl1Δ* mutants, we propose that while heat stress may have general effects on cell permeability, restrictive temperatures may impair the function of Arl1p. Future research may continue to explore the relationship between temperature and ion sensitivity in *S. cerevisiae*. Given that we only tested two temperature conditions, future research may investigate the effects of higher and lower temperatures to determine the range of temperatures at which cells can tolerate ion stressors. Furthermore, future experiments may investigate the molecular mechanisms involved in ion sensitivity. Due to limitations in resource availability, we quantified cell concentrations as a measure of cell growth. Given that there may be a link between the impaired autophagy pathway in *arlΔ* mutants and the observed changes in cell growths with different treatments, future studies may explore the effects of ion sensitivity on autophagy.

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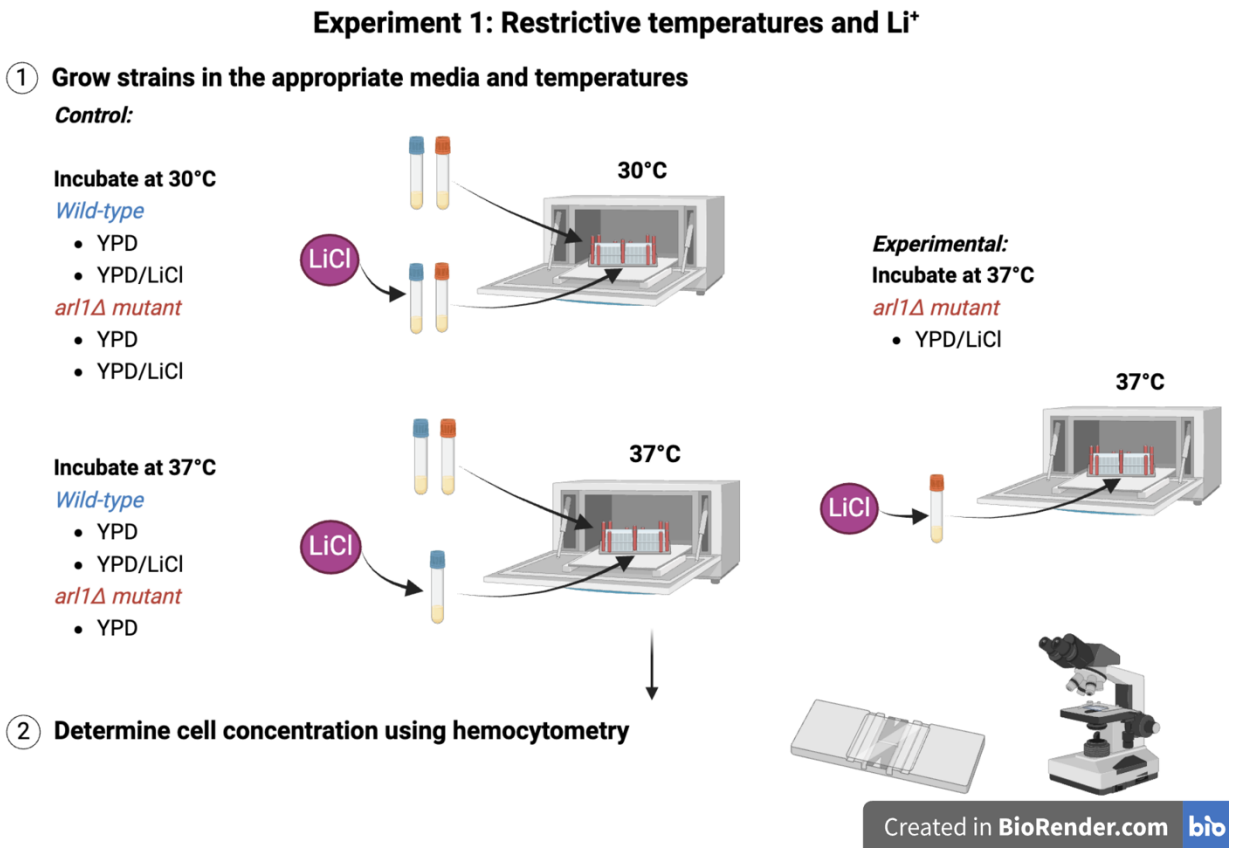
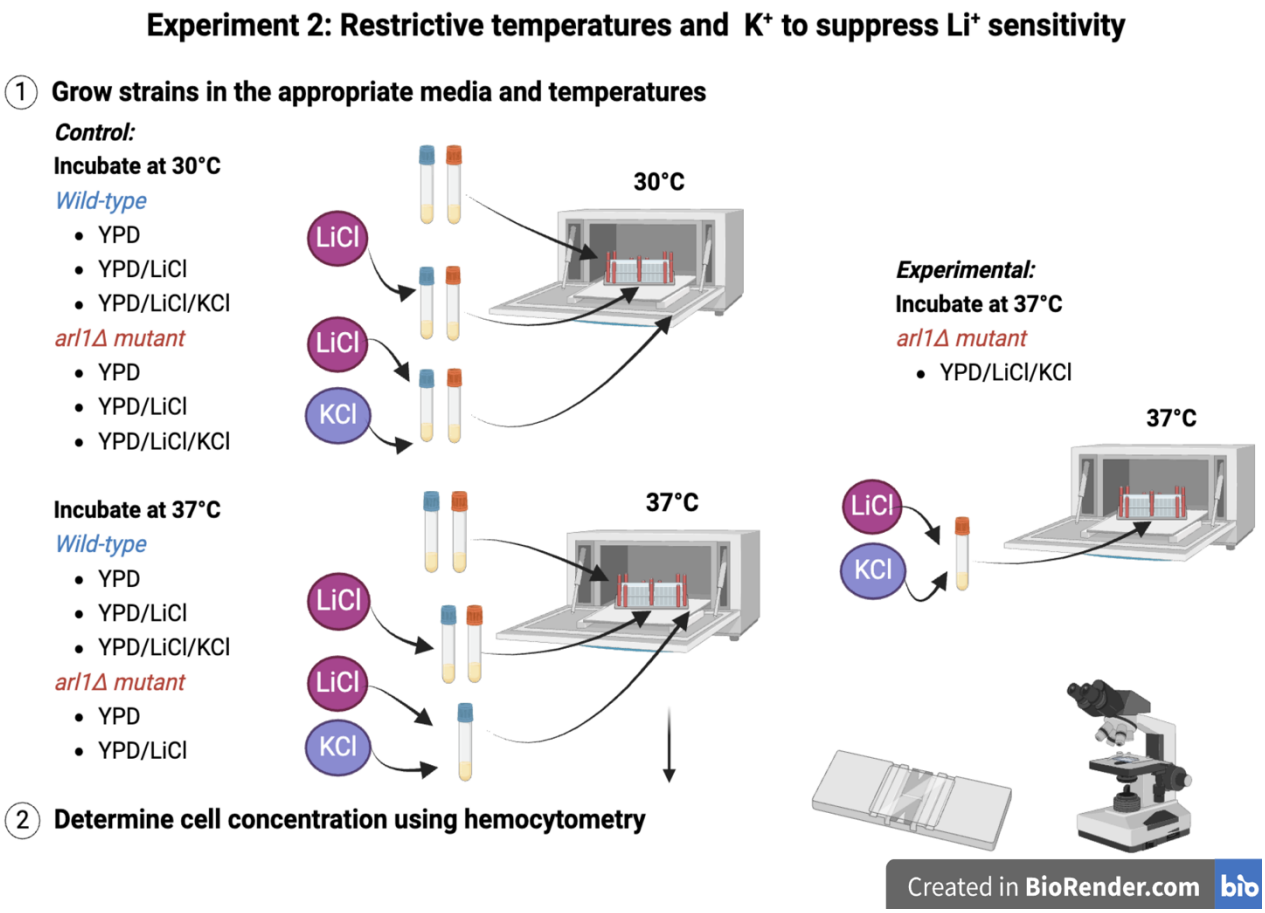
Appendix



Supplementary Figure 1. Flow chart of the procedure of Experiment 1 for the investigation of restrictive temperatures on Li^+ sensitivity. Figure was created using BioRender.com.



Supplementary Figure 2. Flow chart of the procedure of Experiment 2 for the investigation of restrictive temperatures on the suppression of Li^+ sensitivity in the presence of K^+. Figure was created using BioRender.com.