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The *ICL1* and *MLS1* Genes, Integral to the Glyoxylate Cycle, are Essential and Specific for Caloric Restriction-Mediated Extension of Lifespan in Budding Yeast

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The regulation of complex energy metabolism is intricately linked to cellular energy demands. Caloric restriction (CR) plays a pivotal role in modulating the expression of genes associated with key metabolic pathways, including glycolysis, the tricarboxylic acid (TCA) cycle, and the glyoxylate cycle. In this study, the chronological lifespan (CLS) of 35 viable single-gene deletion mutants under both non-restricted and CR conditions, focusing on genes related to these metabolic pathways is evaluated. CR is found to increase CLS predominantly in mutants associated with the glycolysis and TCA cycle. However, this beneficial effect of CR is not observed in mutants of the glyoxylate cycle, particularly those lacking genes for critical enzymes like isocitrate lyase 1 (*icl1*Δ) and malate synthase 1 (*mls1*Δ). This analysis revealed an increase in isocitrate lyase activity, a key enzyme of the glyoxylate cycle, under CR, unlike the activity of isocitrate dehydrogenase, which remains unchanged and is specific to the TCA cycle. Interestingly, rapamycin, a compound known for extending lifespan, does not increase the activity of the glyoxylate cycle enzyme. This suggests that CR affects lifespan through a distinct metabolic mechanism.

1. Introduction

Energy metabolism is essential for the growth, reproduction, and survival of living organisms. Cells produce a universal energy currency adenosine triphosphate (ATP) through energy metabolism including glycolysis, oxidative phosphorylation, and the tricarboxylic acid (TCA) cycle. Regulation of these metabolisms highly depends on nutrition availability and carbon source.[1] Therefore, energy metabolism and sensing pathway are closely connected with lifespan and longevity.[2–4] The depletion of ATP induces apoptosis and necrosis, and these events can be a significant cause of degenerative diseases in the tissues requiring high energy demands including the brain, muscle, and heart.[5,6]

The TCA cycle in mitochondrfia plays a central role in energy metabolism. It provides energy (ATP, NADH, and $FADH_2$), amino acid backbone, and other intermediate metabolites. These metabolites also play an important role in the cellular process, also. Among the TCA cycle intermediates, α-ketoglutarate is a key cofactor for epigenetic modification via Jumonji C domain-containing DNA and histone demethylase, while the accumulation of succinate and fumarate inhibits these enzymes.[7] Therefore, TCA cycle intermediates have been suggested as potential regulators for the hypomethylation of DNA and histone tail during the aging process.[8,9] In addition, an extended lifespan was observed in the worm model by supplementation of malate, fumarate,[10] oxaloacetate,[11] and α-ketoglutarate.[12]

The glyoxylate cycle is the shunt pathway of the TCA cycle in yeast and consists of five enzymes with which overlap three of the five enzymes (aconitase: ACO; citrate synthase: CS; and malate dehydrogenase: MDH) with the TCA cycle and the two key enzymes of the glyoxylate cycle (isocitrate lyase: ICL and malate synthase: MLS). The glyoxylate cycle enables to utilize the non-fermentable carbon sources such as fatty acid, acetate, and ethanol, and the reaction is occurred in both cytosol and peroxisomes.[13] The pathway is required for the survival of bacteria against oxidative stress, and desiccation tolerance of worm and yeast through metabolic rewiring.[14,15] Especially, bacteria, fungi, and plant consist of cell wall, and its major component is polysaccharide.[16–18] For example, in *Saccharomyces cerevisiae*, when the dry weight of cell wall is measured, more than 50% of cell wall is composed of glucan and 35% is mannoproteins which are often heavily glycosylated.[19] Therefore, these organisms require a higher amount of glucose to construct cells than

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organisms without cell walls. In the TCA cycle, it contains two decarboxylation steps which result in the loss of two carbons. For this reason, TCA cycle cannot utilize the C2 compound (especially, acetate from fatty acid beta-oxidation) as a carbon source for gluconeogenesis. Unlike TCA cycle, glyoxylate cycle omits the decarboxylation, allowing the C2 compound to be used as a carbon source for gluconeogenesis.[20] Furthermore, higher glyoxylate cycle enzyme activity was observed in the long-living cell population in heterogeneous yeast cells during chronological aging.[3] Although the glyoxylate cycle has been mainly studied in plant, bacteria, and fungi, major glyoxylate cycle enzyme activity was also detected in worm and mammalian liver tissue.[21] However, the role of the glyoxylate cycle and enzymes in the latter systems has not been well studied.

Our previous study identified the caloric restriction (CR)-dependent key metabolic pathways, including glycolysis, oxidative phosphorylation, and TCA cycle via gene expression profiling in yeast.[22,23] We further studied the role of oxidative phosphorylation genes, and mitochondrial respiration was required for CR-induced lifespan extension.[24] In this study, we identified two other key genes in the glyoxylate cycle for longevity mechanisms.

2. Results

2.1. Differential Effects of CR and Rapamycin on Gene Expression for Energy Metabolism

In a prior study, we delineated the gene expression profiles changed by CR and rapamycin in the BY4741 yeast strain.[22] Our findings revealed that CR significantly modified the expression of genes involved in energy metabolism including glycolysis, the TCA cycle, and oxidative phosphorylation. Conversely, rapamycin exerted negligible effects on the expression of genes in these pathways. These observations suggest that transcriptional alterations of genes in energy metabolism are integral to the longevity mechanisms induced by CR. Specifically, we investigated genes involved in oxidative phosphorylation through the use of mutants for each component of the electron transport chain (ETC). Our results confirmed that functional oxidative phosphorylation is essential for CR-mediated lifespan extension.[24] To further explore potential target genes within CR-associated energy metabolism, we re-examined the gene expression profiles of genes in glycolysis, the TCA cycle, and the glyoxylate cycle (**Figure 1**). Most of these genes were markedly downregulated by CR, whereas rapamycin had a minor or no effect on their expression, indicating a distinct role for energy metabolism in the CR-induced longevity mechanism.

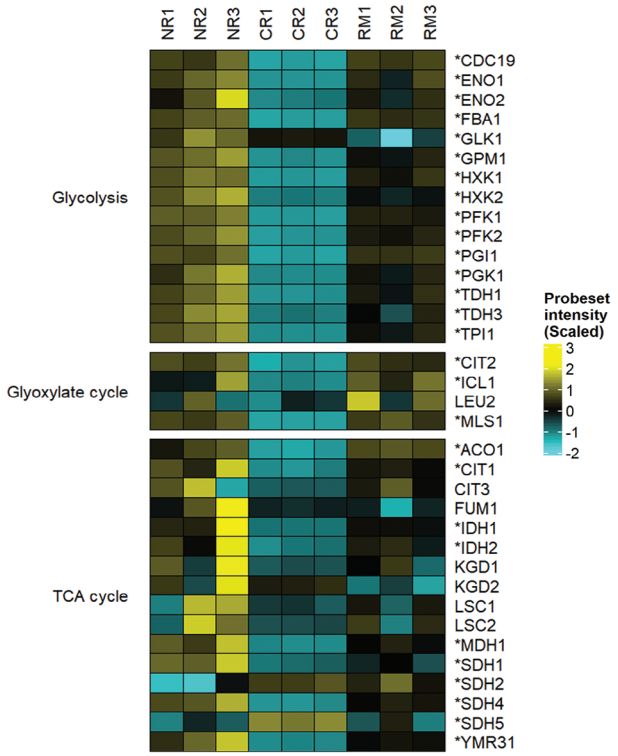


Figure 1. Gene expression profile of genes for energy metabolism under caloric restriction (CR) and rapamycin (RM) treatment in budding yeast. Heat map of gene expression patterns for non-restriction (NR; 2% glucose), CR (0.5% glucose), and 100 nM RM treatment ($n = 3$). The heat map was created based on the DNA microarray data reported in our previous study.[22] Asterisks indicate genes that are differentially expressed.

2.2. Impact of Single-Gene Deletions in Glycolysis, the TCA Cycle and the Glyoxylate Cycle on Chronological Lifespan (CLS)

Eukaryotic cells produce energy in the form of ATP and NADH through catabolic pathways, including glycolysis and the TCA cycle. These pathways are facilitated by several evolutionarily conserved enzymes from yeast to humans. To investigate the genetic underpinnings of major metabolic pathways influencing aging and CR-induced lifespan extension, we measured the CLS of 35 single-gene deletion mutants, non-essential for survival, involved in glycolysis, the TCA cycle, and the glyoxylate cycle under NR (2% glucose) and CR (0.5% glucose) conditions. We found that deletion mutants, *hxk1*Δ, *cit2*Δ, *cit3*Δ, *idh1*Δ, *idh2*Δ, *kgd1*Δ, *kgd2*Δ, *lpd1*Δ, *lsc2*Δ, *sdh1*Δ, *sdh2*Δ, *sdh4*Δ, *fum1*Δ, *and mdh1*Δ strains showed significantly decreased CLS compared to WT under NR condition (**Table 1**; gene names bolded in **Figure 2**). CR led to increased CLS in 31 of the 35 KO strains; however, the CLS of *pfk1*Δ, *cit1*Δ, *icl1*Δ, and *mls1*Δ strains did not extend under CR (Table 1; gene names in red in Figure 2). Notably, while one-third of the glycolytic genes, including *PGI1*, *FBA1*, *PGK1*, *GPM1*, and *CDC19*, were essential for survival, deletion of other non-essential glycolytic genes did not change CLS under NR condition, except for the *hxk1*Δ strain. Interestingly, CR was effective in extending the lifespan for these mutants, except for the *pfk1*Δ strain (Table 1 and Figure 2A).

In the TCA cycle, the deletion of genes encoding enzymes for steps from isocitrate to malate was observed to extend the lifespan in CR media (Figure 2B). Notably, under NR condition, the CLS was significantly reduced in *idh1*Δ, *idh2*Δ, *kgd1*Δ, *kgd2*Δ, *lpd1*Δ, *lsc2*Δ, *sdh1*Δ, *sdh2*Δ, *sdh4*Δ, *fum1*Δ, *and mdh1*Δ strains; however, CR worked in these deletion strains and increased lifespan just like in WT (Figure 2B). Conversely, *icl1*Δ and *mls1*Δ strains, which are involved in the glyoxylate cycle, did not show

Table 1. Two-way ANOVA *p*-value of lifespan difference between wild type and knock out mutant or between NR and CR within knock out mutant. *p* values were obtained from a two-way ANOVA with time (days) and strain or time (days) and media conditions, as independent factors. Text in bold represents CLS significantly different between wild type and mutant strain ($p < 0.01$) for WT versus KO, or loss of CR effect in mutant strain ($p > 0.01$) for NR versus CR.

Pathway	Gene name	WT vs KO	NR vs CR
glycolysis	hxk1	**0.007**	0.000
	hxk2	0.641	0.002
	glk1	0.065	0.000
	pfk1	0.014	**0.126**
	pfk2	0.710	0.000
	tdh1	0.159	0.000
	tdh2	0.571	0.001
	tdh3	0.221	0.000
	eno1	0.373	0.000
	eno2	0.034	0.000
	pyk2	0.031	0.000
TCA cycle	cit1	0.284	**0.487**
	cit2	**0.007**	0.000
	cit3	**0.000**	0.000
	aco2	0.342	0.000
	idh1	**0.000**	0.000
	idh2	**0.000**	0.000
	kgd1	**0.000**	0.000
	kgd2	**0.000**	0.000
	lpd1	**0.000**	0.000
	lsc1	0.178	0.000
	lsc2	**0.002**	0.000
	sdh1	**0.000**	0.000
	sdh2	**0.000**	0.000
	sdh4	**0.000**	0.000
	frd1	0.163	0.002
	osm1	0.057	0.000
	fum1	**0.000**	0.000
	mdh1	**0.000**	0.000
	mdh2	0.116	0.000
	mdh3	0.096	0.000
	idh1idh2	0.000	0.000
glyoxylate cycle	icl1	0.562	**0.020**
	icl2	0.316	0.000
	mls1	0.342	**0.137**
	dal7	0.531	0.000
	icl1icl2	0.315	**0.327**

lifespan extension under CR (Figure 2B). Intriguingly, deletion strains of *ICL2* and *DAL7*, which are homologs of *ICL1* and *MLS1*, respectively, did not mimic the phenotypes of their paralogs. It is important to note that these genes also participate in other metabolic pathways, specifically the methyl citrate cycle and allantoin degradation, respectively, as described in other studies.[25,26]

2.3. The Glyoxylate Cycle was Required for CR-Mediated Lifespan Extension

To further explore the specific roles of the intact glyoxylate and TCA cycles in mediating the effects of CR, we generated double deletion mutants, *icl1*Δ*icl2*Δ and *idh1*Δ*idh2*Δ, which block the initial step of the glyoxylate cycle and a TCA cycle-specific step, respectively. In these mutants, CR failed to extend the CLS of the *icl1*Δ*icl2*Δ strain, whereas it successfully increased the CLS of the *idh1*Δ*idh2*Δ strain (Table 1 and **Figure 3**).

Subsequently, to assess whether enzyme activities of the glyoxylate and TCA cycles are differentially affected by CR, we measured the activities of ICL and NAD^+ dependent IDH (isocitrate dehydrogenase) during chronological aging process under both NR and CR conditions. Both enzymes use isocitrate as a substrate, competing for it in their respective cycles. While CR significantly enhanced ICL enzyme activity (**Figure 4A**), IDH enzyme activity remained unchanged (Figure 4B). These findings underscore the critical role of the glyoxylate cycle in facilitating lifespan extension via CR.

2.4. Rapamycin did not Enhance the Glyoxylate Cycle Enzyme Activity

Rapamycin is a well-known pharmacological agent that extends lifespan by inhibiting the target of rapamycin (TOR) in various species, including yeast,[27] worm,[28] fly,[29] and mouse.[30] Given that CR is also known to modulate the TOR/SCH9 pathway, many researchers have proposed that CR and rapamycin might share similar longevity mechanisms.[31] To explore whether rapamycin also increases glyoxylate cycle activity like CR, we administered 100 nM rapamycin, previously shown to maximize lifespan extension,[22] and measured ICL and IDH enzyme activities during the stationary phase. Contrary to CR, rapamycin did not significantly change ICL and IDH activities (**Figure 5**). Consequently, while both CR and rapamycin extend lifespan, they appear to do so via distinct metabolic processes, with the glyoxylate cycle enhancement being specific to CR-induced lifespan extension. This aligns with our earlier findings and recent studies highlighting differences in the longevity mechanisms between CR and rapamycin.[22,32]

3. Discussion

Glycolysis, the TCA cycle, and the ETC are fundamental metabolic pathways that produce energy molecules such as ATP, NADH, and $FADH_2$. These pathways are intricately linked to ensure cellular functionality and survival. Particularly, the ETC has been identified as a crucial pathway for lifespan maintenance.[24,33] To further understand the role of energy metabolism in longevity, we examined the effects of single and double-gene KOs in glycolysis, the TCA cycle, and the glyoxylate cycle on the aging process. Our findings revealed that the glyoxylate cycle is necessary for CR-induced lifespan extension. Furthermore, the activity of the ICL enzyme, which catalyzes the initial step of the glyoxylate cycle, was elevated by CR, whereas the activity of the IDH enzyme, a TCA cycle-specific enzyme, remained

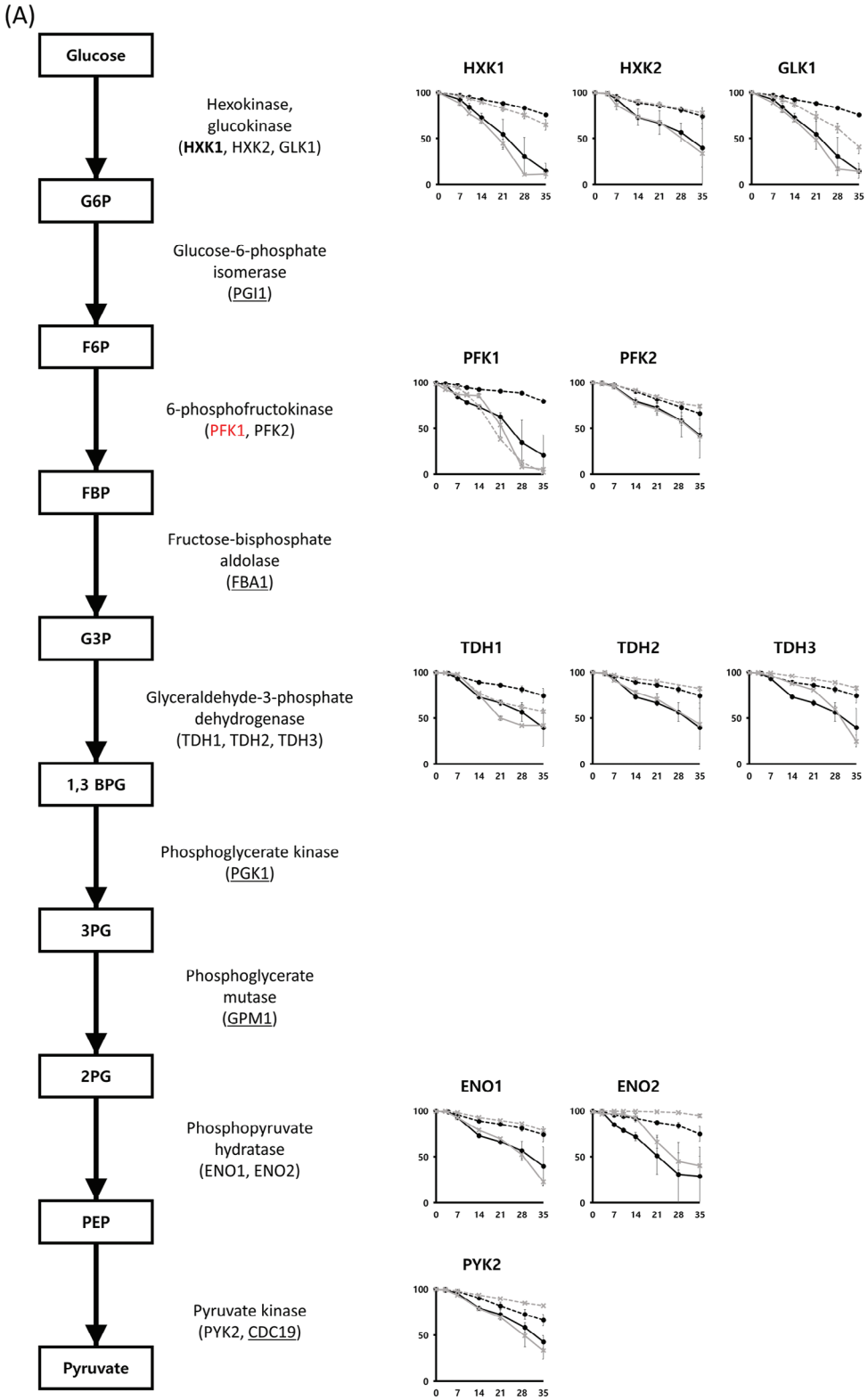
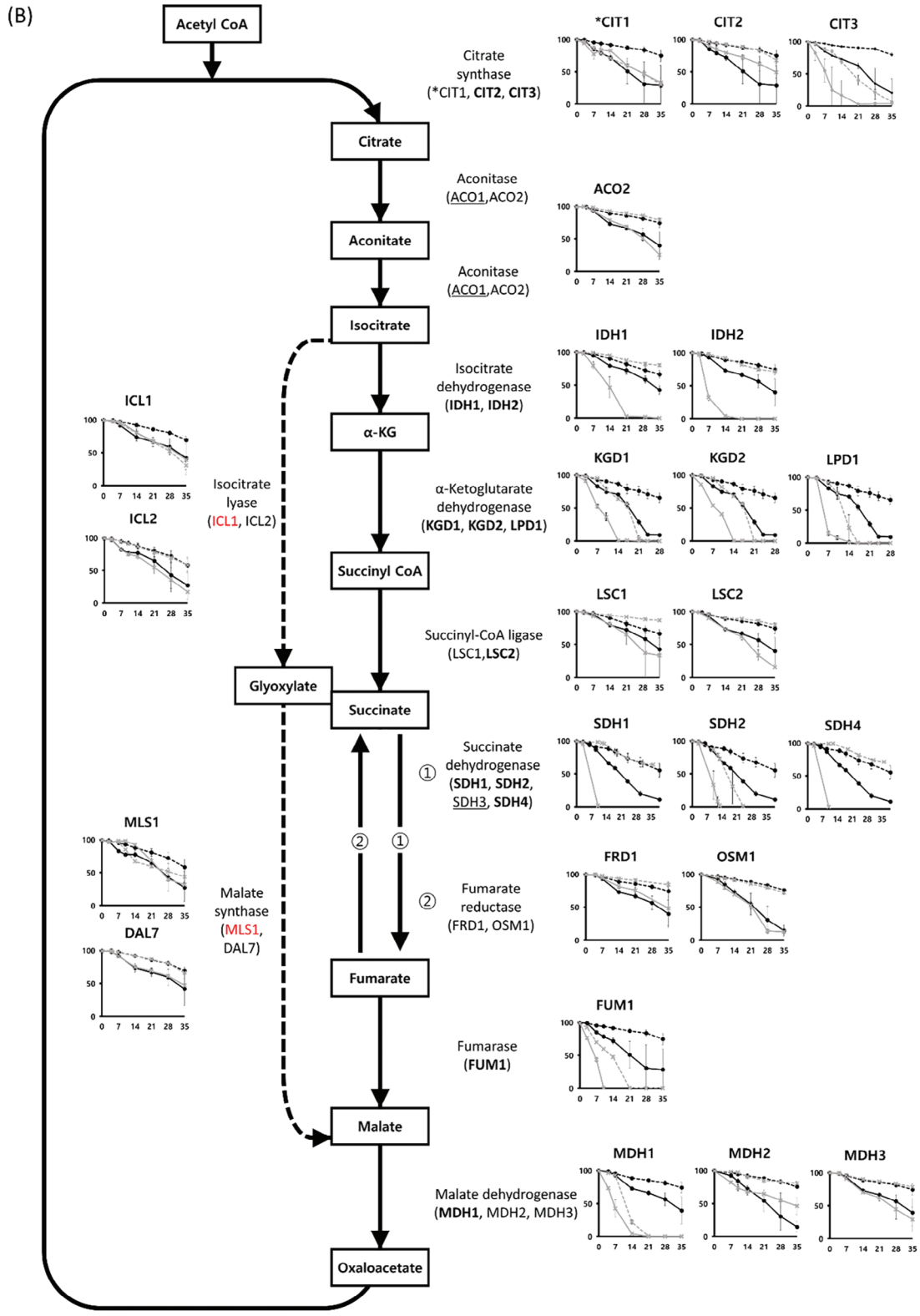


Figure 2. Chronological lifespan (CLS) of single-gene deletion mutants for glycolysis A) and TCA cycle B) under NR and CR conditions. CLS of single-gene deletion mutants for the glycolysis pathway A) and TCA cycle B) was measured by propidium iodide (PI) staining ($n = 3$). Genes marked in red represent the mutants that did not exhibit CR-induced lifespan extension, and genes in bold represent the mutants where lifespan was shortened compared to the wild type. Underlined genes are essential for survival. In the CLS graphs, the horizontal axis (x-axis) indicates a time in days, and the vertical axis (y-axis) shows the viability of cells (black line for wild type, WT, and grey line for knock-out, KO, biological triplicate; error bars indicate standard deviation).



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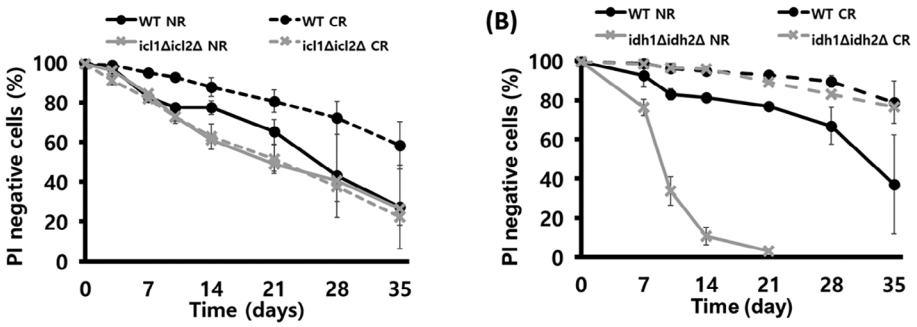

Figure 3. Blocking the glyoxylate cycle and TCA cycle under NR and CR conditions. CLS of double KO (DKO) strains: isocitrate lyase (ICL) genes (*icl1*Δ*icl2*Δ) A) and isocitrate dehydrogenase (IDH) genes (*idh1*Δ*idh2*Δ) B). Error bars represent standard deviation ($n = 3$).

unchanged (Figure 4). These findings suggest that CR preferentially enhances the glyoxylate cycle over the TCA cycle to confer health benefits in energy metabolism.

The glyoxylate cycle is a branch pathway of the TCA cycle, and this pathway was shunted from the isocitrate in the TCA cycle. ICL and IDH utilize the isocitrate as a substrate for the glyoxylate and TCA cycles, respectively. These enzymes compete for available isocitrate in cells. IDH has a higher substrate affinity for isocitrate than ICL. Therefore, a decline in IDH substrate-binding affinity, such as phosphorylation of IDH at Ser 113, is required to enhance ICL enzyme activity.[34]

The glyoxylate cycle and TCA cycle share enzymes including MDH, CS, and ACO. Yeast has three CS isoenzymes, including mitochondrial CS encoded in *CIT1* and *CIT3*, and peroxisomal CS encoded in *CIT2*.[35–37] Among these enzymes, only *CIT1* was required for CR-induced lifespan extension (Figure 2). Peroxisomal CS, *CIT2* is known to have a function in the glyoxylate cycle, but *CIT1* is not.[38] In the yeast model, the ethanol-dependent glyoxylate cycle occurs in the cytosol during respiration whereas the fatty acid-dependent glyoxylate cycle occurs in the peroxisome.[13] Mutation of *CIT1* impairs glyoxylate cycle-dependent acetate metabolism in yeast.[39] The yeast produces non-fermentable carbon sources such as ethanol and acetate as byproducts from glucose fermentation, and the cells switch their metabolism to utilize these non-fermentable carbon sources. Malate synthase (MS) also has two isoenzymes with different localization, however, their deletion does not affect CR-induced lifespan extension (Figure 2).

Rapamycin, an inhibitor of TOR, is the first drug that is known to extend lifespan in yeast,[40] worms,[41] flies,[29] and rodents.[42] Rapamycin has been studied as a CR mimetic because both CR and rapamycin inhibit the TOR signaling pathway and have a similar effect on some processes.[43] However, the difference in the detailed mechanisms of CR and rapamycin is still uncertain. In our previous study, rapamycin enhanced the breakdown pathways of energy storage molecules such as glycogen and lipid droplets whereas CR did not.[22] Therefore, CR and rapamycin may differentially affect energy metabolism. As expected, CR enhanced the glyoxylate cycle to achieve lifespan extension whereas rapamycin did not alter either the TCA or glyoxylate cycle (Figure 5). These findings demonstrate that CR and rapamycin exhibit distinct longevity mechanisms and that the increased glyoxylate cycle activity is a specific longevity mechanism of CR.

Energy metabolism consists of the main metabolic pathways and various branch pathways. They are tightly regulated by available sources of energy and cellular energy demands. Consequently, these metabolic changes affect energy efficiency for cellular survival and longevity. However, the detailed role of a branch metabolic pathway and communication with other metabolic pathways has not been easily revealed due to the complexity of the energy metabolism. In this study, we identified that a branch pathway of the TCA cycle, the glyoxylate cycle, was enhanced by CR and required for CR-induced lifespan extension, whereas the TCA cycle was not. Our findings showed that the metabolic pathway was remodeled alongside the metabolic branch pathway depending on nutrient availability, and eventually influenced cellular longevity.

Unlike other organisms, bacteria, fungi, and plants have cell walls, which are composed of cellulose, chitin, glucan, and other components.[16–18] Especially, more than 50% of the cell wall in Saccharomyces cerevisiae consists of glucan, a polysaccharide derived from glucose.[19] Therefore, it may be difficult to maintain the cell wall intact because glucose is limited in CR conditions. To overcome this circumstance, the glyoxylate cycle can serve as an alternative. In the TCA cycle, there are two decarboxylation steps which result in the loss of two carbons, so it is impossible to utilize C2 compound, such as acetate from fatty acid beta-oxidation, as a carbon source for gluconeogenesis. However, the glyoxylate cycle bypasses the decarboxylation step of the TCA cycle, and produces malate and oxaloacetate from acetyl-CoA. Then, these products can be converted into phosphoenolpyruvate, which is the substrate for the gluconeogenesis pathway.[20] In other words, utilizing the C2 compound as gluconeogenesis source through glyoxylate cycle can improve cell wall maintenance of yeast in CR condition, which could be the reason why glyoxylate cycle enzymes are essential for CR-mediated life span extension.

The glyoxylate cycle has been known as a specific shunt pathway that occurs in plants, bacteria, and fungi. Recently, the glyoxylate cycle in higher organisms including *C. elegans*,[15] birds[44] and mammals, especially in their liver, was reported.[45] Although there is no evidence for the presence of the glyoxylate cycle in humans, malate synthase and β-methylmalate synthase activities have nevertheless been reported.[46] Moreover, variations of the TCA cycle have been suggested in higher organisms, and aberrant TCA cycle enzymes are known to participate in diverse pathological processes such as cancer and obesity.[47,48] Along with this evidence, our results suggest that the glyoxylate

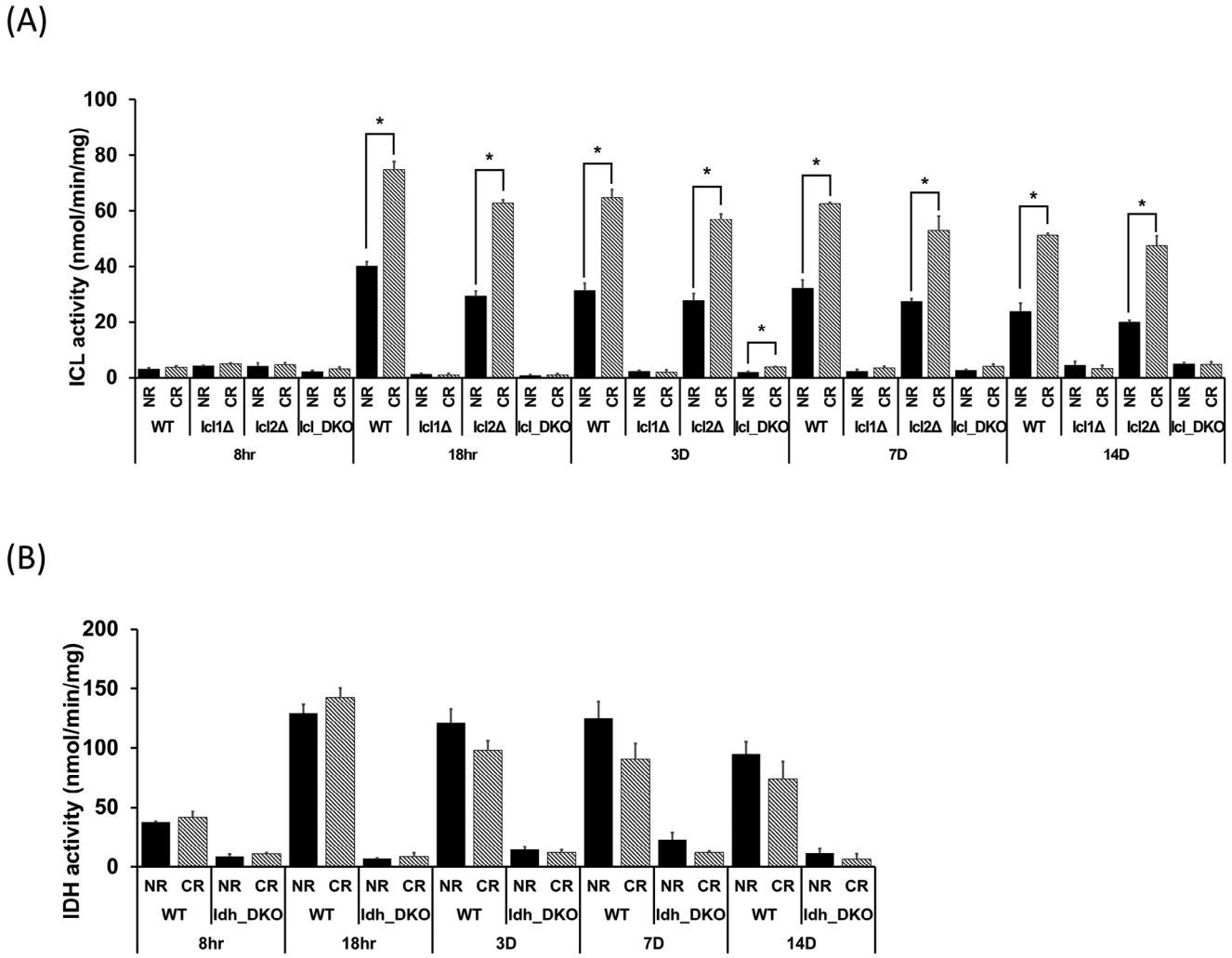

Figure 4. ICL and IDH enzyme activity during chronological aging under NR and CR conditions. A) ICL enzyme activity of WT and mutants; B) IDH enzyme activity of WT and IDH DKO during the chronological aging process. Error bars represent standard deviation ($n = 3$). *Significant difference from NR ($p < 0.01$; Student's *t*-test, two-tailed).

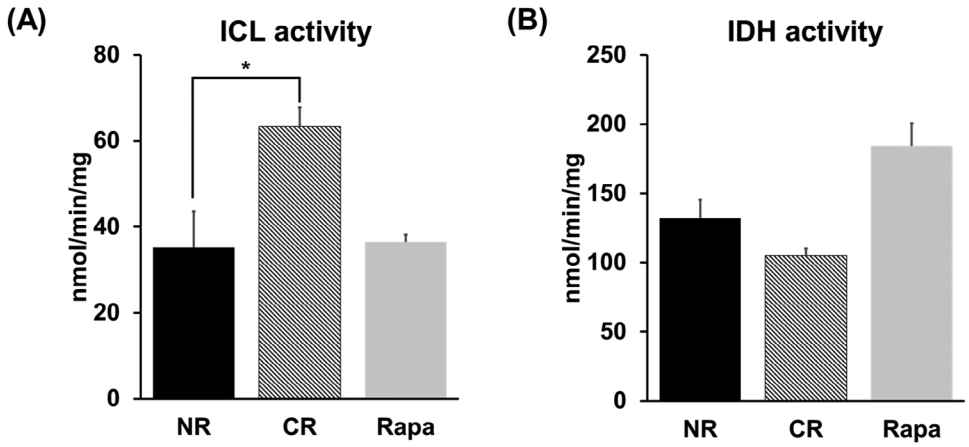

Figure 5. ICL and IDH enzyme activities by CR and rapamycin treatment. ICL and IDH activities were measured at day 5 under NR, CR, and 100 nM rapamycin treatment. Error bars represent standard deviation ($n = 3$). *Significant difference from NR ($p < 0.01$, Student's *t*-test, two-tailed).

cycle-associated CR-induced lifespan extension implies that CR-induced health benefits and longevity mechanisms in mammals also may be associated with a switch in the energy metabolic pathway through the glyoxylate cycle-like shunt pathway. In a recent study, the treatment of glyoxylate conferred resistance to cyanide toxicity in mouse, rabbit, and fish.[49] Also, glyoxylate enzymes (ICL and MS) have been detected in the liver of alloxan-treated rats.[50] Perhaps, just as the cell wall maintenance pathway in yeast has evolved into angiogenesis in humans,[51] the enzymes in the yeast glyoxylate cycle might have evolved into chemical resistance in mammals.

4. Experimental Section

Yeast Strains and Culture Conditions: Isogenic single-gene deletion strains were obtained from the BY4741 (*MATa his3Δ1 leu2Δ0 met15Δ0, ura3Δ0*) genetic background of *Saccharomyces cerevisiae*. The 40 glycolysis, TCA cycle, and glyoxylate cycle gene deletion strains (*glk1Δ, hxk1Δ, hxk2Δ, pfk1Δ, pfk2Δ, tdh1Δ, tdh2Δ, tdh3Δ, eno1Δ, eno2Δ, pyk2Δ, pda1Δ, pdb1Δ, pyc1Δ, pyc2Δ, mdh1Δ, mdh2Δ, mdh3Δ, cit1Δ, cit2Δ, cit3Δ, aco2Δ, idh1Δ, idh2Δ, kgd1Δ, kgd2Δ, lpd1Δ, lsc1Δ, lsc2Δ, frd1Δ, oxm1Δ, sdh1Δ, sdh2Δ, sdh4Δ* and *fum1Δ*) from the Open Biosystems Yeast library were genotyped using PCR-based methods.

Yeast strains were streaked onto YPD agar plates (2% Bacto agar, 1% Bacto yeast extract, 2% Bacto peptone, and 2% Difco dextrose (BD Diagnostics, CA, USA)). Plates were incubated at 30 °C until single colonies appeared. Isolated single colonies were inoculated into 10 mL of YPD medium containing 1% Bacto yeast extract, 2% Bacto peptone, and 2% Difco dextrose (BD Bioscience, USA), followed by overnight cultured. This overnight seed culture was inoculated into 10 mL of fresh 2% YPD medium and cultured for 10 min. Subsequently, the final seed culture was inoculated into 20 mL of 2% or 0.5% glucose-containing YPD medium for NR and CR conditions, respectively. The cultures were adjusted to an initial $OD_{600} = 0.05$. All yeast cultures were incubated in a 30 °C orbital shaking incubator at 200 rpm.

Construction of Double Gene Mutant Strains: DKO mutants of isocitrate lyase and isocitrate dehydrogenase were obtained from *icl1* and *idh1* KO strains in the yeast deletion library, respectively. The mutants were constructed by homologous recombination gene editing using pRS406 plasmid (ATCC, cat#87517) as the previously described method.[3]

Measurement of CLS: Assessment of CLS using PI was performed as previously described.[3] Yeast cells were harvested by centrifugation and resuspended with 1 mL phosphate-buffered saline (PBS) for washing. Washed cells were incubated for 20 min at 30 °C in 5 μg mL^{-1} PI solution (Sigma Aldrich, St. Louis, MO, USA). Stained cells were analyzed with flow cytometry (FACS Verse; Becton Dickinson, NJ, USA). Excitation was performed using a blue laser at 488 nm and emission was detected at 585 nm. A total of 20 000 cells were analyzed for each sample, and data were analyzed using Cell Quest software (Becton Dickinson).

Isocitrate Lyase and Isocitrate Dehydrogenase Enzyme Activity: Isocitrate lyase enzyme activity was measured according to Chell, R.M (1978). with minor modifications.[52] Harvested cells were washed twice and resuspended in lysis buffer (25 mM potassium phosphate, 1 mM EDTA, 0.15 M KCl, 0.2 mM PMSF at pH 7.0). The cells were transferred glass bead contained a screw cap tube and homogenized by a beadbeater (Mini-Beadbeater 16, Biospec, OK, USA). The supernatant was collected by centrifugation as isocitrate lyase enzyme solution. The samples were mixed with isocitrate lyase activity assay buffer (12.5 mM potassium phosphate buffer (pH 6.8), 5 mM MgCl2, 1 mM EDTA, 4 mM Phenylhydrazine HCl, and 1 mM isocitrate as a final concentration). The absorbance value at 324 nm was measured every minute for 30 min by Multi-detection microplate reader at 30 °C with shaking.

Statistical Analysis: Gene expression data using DNA microarray were re-investigated from the previous report (Choi et al., 2017). Previously identified 35 genes involved in glycolysis, the glyoxylate cycle, and the TCA cycle were visualized using the Heatmap function in the R ComplexHeatmap package.

For CLS data, *p*-values were obtained from a two-way analysis of variance (ANOVA) with time (days) and strain or with time (days) and media conditions used as independent factors using the aov function in the R stat package. For ICL and IDH enzyme activity data, *p*-values were determined by Student's *t*-test using the t.test function in the stats package. When *p*-values lower than 0.01 are considered statistically significant. The R version 4.1.0 (R Core Team, Austria) was used for all statistical analyses. Data with error bars are represented as the mean ± SD, with n = 3 (biologically independent triplicates) for each experiment.

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Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

YK and HL contributed equally to this study. YL and CL conceptualized the project and planned experiments. YK, HL, and ML performed experiments. YK and HL processed data, analyzed data, and prepared figures. YK, HL, YL, and CL drafted the manuscript, and YL and CL finalized the manuscript. All authors approved the manuscript.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Keywords

caloric restriction, glyoxylate cycle, longevity, rapamycin

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