CALORIE RESTRICTION EXTENDS LIFESPAN IN YEAST INDEPENDENTLY OF *GCN5*

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ABSTRACT

Calorie restriction (CR) has been shown to increase longevity in various animals; however, the mechanisms by which it affects chromatin-modifying factors remain poorly understood. *GCN5*, a histone acetyltransferase, is a vital regulator of transcription that responds to metabolic and stress signals, linking nutrient signaling to chromatin remodeling, but the extent of its necessity in promoting longevity through caloric restriction (CR) has yet to be completely defined. We investigated chronological lifespan (CLS), growth dynamics, respiratory efficiency, oxidative stress tolerance, mitochondrial DNA (mtDNA) mutation rate, and mtDNA abundance in a yeast *gcn5Δ* mutant under both normal and glucose-restricted conditions in order to elucidate *GCN5*-independent effects of CR. Under CR, the mutant showed a significant increase in CLS along with a decreased growth rate and improved survival in the stationary phase. Despite the loss of *GCN5*, calorie restriction enhanced respiratory efficiency on non-fermentable substrates, improved survival under oxidative stress, increased mtDNA abundance, and reduced mtDNA mutations, suggesting improved mitochondrial integrity. Altogether, these results showed that CR can compensate for *GCN5*'s absence by triggering alternative nutrient-sensing and mitochondrial maintenance systems. This information offers insights into chromatin-independent pathways that could be used to mitigate aging and mitochondrial dysfunction in higher organisms

KEYWORDS: Yeast, Calorie restriction, *GCN5*, Aging and Mitochondria

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Introduction

Calorie restriction (CR) is a dietary approach that involves decreasing energy consumption by approximately 25–30% while maintaining essential nutrient levels (Diet, 2021). The most common technique for mimicking calorie restriction in yeast cells is the depletion of glucose (Taormina and Mirisola, 2014). Cell metabolism relies on glucose, which also influences cellular physiology. Therefore, its availability can significantly influence the rate of cell growth, metabolism, and the generation of cellular byproducts such as reactive oxygen species. Yeast is among the various organisms that demonstrate the advantages of calorie restriction in environments with low glucose levels. Genetic modification (alteration of genes associated with glucose signaling) and calorie restriction could affect the morphological and physiological traits of the yeast cell (Maslanka, Kwolek-Mirek and Zadrag-Tecza, 2017).

CR extends yeast's lifespan by quiescence induction (Jiménez *et al.*, 2015), slowing growth (Leonov *et al.*, 2017), increased respiration (Kwon, Lee and Lee, 2017), mitochondrial structure and function maintenance (Lanza *et al.*, 2012), increasing levels of mitochondrial DNA (Kwon, Lee and Lee, 2017) as well as remodeling carbohydrate and lipid metabolism (Arlia-ciommo *et al.*, 2018).

CLS, or chronological life span, measures how long yeast cells survive in a non-dividing state. Since most cells leave the cell cycle during the post-diauxic phase, CLS is typically measured by allowing a yeast culture to reach this stage. Approximately 24 hours following the initial inoculation, the post-diauxic phase begins. Chronological aging in yeast is regulated by pathways that detect nutrient availability and control their usage, notably the Ras/adenylate cyclase (AC)/PKA pathway, which is chiefly triggered by glucose (Longo, 2013).

The availability of nutrients partially regulates yeast growth and proliferation (Werner-washburne *et al.*, 1993). In nutrient-rich environments, young cells proliferate rapidly until they encounter the diauxic shift at the conclusion of the logarithmic phase (Gregorio and Duennwald, 2021). When yeast runs out of sugar, it enters the stationary phase, and entering this quiescent state typically corresponds to a significant decrease in growth rate (Herman, 2002).

Glucose is the favored carbon source in yeast, and fermentation dominates energy production, even when oxygen is available.

As sugar levels drop, cells begin to utilize ethanol produced during fermentation by switching to respiration. Yeast can also utilize alternative non-fermentable carbon sources (Repressor, 2014). When yeast cells are grown on non-fermentable carbon sources like glycerol, mitochondrial networks become more branched and expand in volume, supporting increased respiratory activity (Bagamery *et al.*, 2020).

S. cerevisiae features a complex oxidative stress defense system that recognizes ROS signals, including hydrogen peroxide (H2O2) (Feng, Yu and Ye, 2025). This system includes numerous sets of enzymes that operate directly as ROS detoxifiers (Okada, Ogawa and Shima, 2014). Among these, peroxiredoxins function as key H_2O_2-scavenging enzymes. In yeast, the cytosolic peroxiredoxin is essential for promoting resistance to H_2O_2 and for mediating lifespan extension in response to caloric restriction (Roger *et al.*, 2020).

Somatic mtDNA point mutations rise with normal ageing because mtDNA is prone to mutation. A mutator phenotype can result from an increase in the frequency of mtDNA point mutations caused by mutations in genes related to mtDNA metabolism (Larsson, 2010). The mutation is most likely the result of damage or loss to the yeast's mitochondrial DNA, since petite mutants frequently have either no mitochondrial DNA at all or abnormally high or low buoyant densities (Whittaker, 1972). Erythromycin antibiotic selectively blocks mitochondrial translation, and resistance to this antibiotic occurs via point mutations in the mitochondrial RNR2 and RNR3 loci encoding mitochondrial rRNAs (Doudican *et al.*, 2005). Interestingly, one of our studies found that calorie restriction reduces the spontaneous mtDNA mutation rate in yeast (Mallick *et al.*, 2025).

There are several copies of the mtDNA molecule in yeast cells. Based on the strain and development circumstances, it has been estimated that each cell has 10–50 to 50–200 mtDNA copies per nuclear genome (Ionut, Martina and Ilenia, 2023). The life cycle and physiological status of the cells are closely related to the quantity, shape, and volume of mitochondria. Whereas quiescent-phase cells have numerous spherical or fragmented mitochondria, log-phase cells have few tubular ones. Thus, mitochondrial morphology is dynamic and changes in response to environmental factors rather than being static (Miyakawa, 2017).

The molecular mechanisms underlying CR-induced lifespan extension involve multiple nutrient-sensing and epigenetic regulators, among which *GCN5* is one. *GCN5*, a histone acetyltransferase involved in nutrient signaling and nutrient sensing, plays a key regulatory role. General Control Non-depressible 5 protein (*GCN5*), produced by the mammalian gene Kat2a, is the first identified histone acetyltransferase that connects histone acetylation to the activation of transcription (Brownell *et al.*, 1996). As part of the HAT module of the SAGA/SLIK complex, *GCN5* regulates the expression of starvation-induced stress-response genes, supports respiratory growth, maintains redox balance, and promotes the accumulation of storage carbohydrates as cells enter the stationary phase, thereby contributing to the extension of chronological lifespan (CLS) (K. Li *et al.*, 2023). *GCN5* activity is closely linked to the cell's metabolic and energy status, enabling it to regulate the expression of genes involved in nutrient sensing and metabolic adaptation. It influences energy metabolism mainly by sensing levels of acetyl-CoA, the key metabolite used in its acetylation reaction, and by acetylating proteins like PGC-1α, a major regulator of metabolic and mitochondrial genes (Mutlu and Puigserver, 2021).

The activity of *GCN5* is dynamically regulated by nutrient status. During carbon starvation, global histone acetylation patterns are redistributed, with fat oxidation and gluconeogenic genes acquiring acetylated histones to enable adaptive metabolic responses. This switch in the histone acetylome is mediated by Gcn5p-containing SAGA complexes (Hsieh *et al.*, 2022). Interestingly, during the feeding-to-fasting transition, *GCN5* exerts a dual function through a glucagon-dependent substrate switch. It activates gluconeogenesis as an HAT in the fasted state and inhibits it via PGC-1α acetylation in the fed state (Sakai *et al.*, 2016). These nutrient-responsive adaptations link *GCN5* to caloric restriction (CR) and stress-response pathways, which are key modulators of longevity.

The nutrient-responsive adaptability of *GCN5* forms the basis for its wider influence on various physiological and cellular functions. In humans, *GCN5*-mediated acetylation is associated with chromatin modification, chromosome condensation, autophagy, neuronal apoptosis, hematopoiesis, oxidative stress, cell proliferation, and stem cell differentiation. Due to its role in epigenetic regulation and the pathophysiology of multiple diseases, such as cancer, diabetes, multidrug resistance, neurological disorders, immune disorders, infectious diseases, and aging, *GCN5* has received significant research attention (Haque *et al.*, 2021).

Although *GCN5*, a histone acetyltransferase, is a central regulator of chromatin remodeling, nutrient signaling, and stress responses, its role in modulating yeast aging under calorie restriction remains poorly understood. In particular, it is unclear whether CR can extend lifespan in the absence of *GCN5* and whether alternative, *GCN5*-independent pathways contribute to CR-mediated longevity. To address this knowledge gap, we investigated the effects of CR on yeast cellular physiology in a *gcn5*Δ background. This study may provide insights into the extent to which CR promotes longevity independently of *GCN5* and help identify potential mechanisms that could guide the development of therapeutic or dietary strategies to mimic CR benefits in individuals with age-related chromatin alterations.

Materials and methods

Yeast strain and media

The genetic background of isogenic deletion mutant yeast strain (*gcn5Δ*) was obtained from EUROSCARF and used in this study. The cells were stored in glycerol stocks at −20 °C and periodically subcultured on YPD plates at 30 °C.

Table 1. Composition of normal and calorie-restricted media used in this study

Media Type	Standard	Calorie restriction
YPD (solid/liquid)	1% yeast extract, 2% peptone, 2% dextrose (solid media contains 2% agar) (Dueñas-Sánchez *et al.*, 2012)	1% yeast extract, 2% peptone, 0.5% dextrose (solid media contains 2% agar) (Lin, Defossez and Guarente, 2000)
YPG (solid/liquid)	1% yeast extract, 2% peptone, 3% glycerol (solid media contains 2% agar) (Dueñas-Sánchez *et al.*, 2012)	1% yeast extract, 2% peptone, 2% glycerol (solid media contains 2% agar) (Wallis and Whittaker, 1974)

Chronological lifespan assay

The ability of non-dividing cultures to remain viable over time is known as yeast chronological life span (CLS), and the colony formation unit test (CFU) is commonly used to assess cellular viability (Ocampo and Barrientos, 2011). For this study, mutant cells were cultured in both standard and calorie-restricted liquid (CR) media in 250 mL conical flasks using a flask-to-media volume ratio of 5:1. Flasks were maintained at 30 °C with agitation at 180 rpm for a period of 30 days. Starting on day two, 1.0 mL samples were taken from each flask, and OD_{600} was measured. Samples were diluted with sterile water to an OD of 0.4 using the formula: $(0.4 \times 1000) / OD_{600}$. Subsequent serial dilutions were performed by adding 5 µL of cell culture to 995 µL of distilled water. Thirty microliters of the diluted cultures were plated on both standard and CR YPD plates and incubated at 30 °C for 48 hours to allow colony formation. Colony-forming units (CFUs) were then counted to determine cell viability for each sample. CFUs were normalized by adjusting every sample to an OD_{600} of 0.4. Therefore, any differences in CFU counts reflect true differences in cell viability. The cells' survival rate was assessed using three replicas by repeating the same procedure every 72 hours for 30 days. To assess differences in lifespan, survival curves were plotted using OASIS (Online Application for Survival Analysis), with Kaplan–Meier analysis for survival percentages and the log-rank test for statistical significance.

Growth curve determination

Both standard and calorie-restricted liquid YPD media were placed in two separate conical flasks, with 100 mL in each. Each flask was inoculated with a single colony of the *gcn5Δ* strain and maintained in a shaking incubator at 30 °C and 150 rpm for 30 days. OD_{600} was measured using 1 mL samples daily for the first 15 days and every three days thereafter until day 30. This experiment was performed with three biological replicates.

Respiration efficiency assay

This experiment was conducted to determine whether the mutant strain's ability to metabolize glycerol as a carbon source differs between cells grown in standard and calorie-restricted (CR) YPG media. Both regular and calorie-restricted liquid YPD and YPG media were used to inoculate the mutant yeast strain. After a two-day incubation at 30 °C, the optical density (OD) was adjusted using the formula $(0.2 \times 1000 / OD_{600})$. The cultures were then serially diluted three times to concentrations of 0.02, 0.002, and 0.0002, and 5 µL of each diluted culture was spotted onto both normal and calorie-restricted YPD and YPG plates. Plates were incubated at 30 °C for 48 hours. This experiment was repeated five times to get a consistent result.

Oxidative stress tolerance assay

After 2 days of culture, equal volumes from both samples were adjusted to an OD_{600} of ~12. The cultures were then centrifuged at 3,900 × g for 3 min, and the supernatant was removed. Then cells were resuspended in 20 mL pre-warmed YPD and vortexed to mix. For each strain, 10 mL of culture was transferred into two sterile flasks, and H_2O_2 was added to one flask to a final concentration of 4 mM; the second flask served as an untreated control. Cultures were incubated at 30 °C for 30 min with shaking. After incubation, 1 mL of each culture was collected, centrifuged at 12,200 × g for 2 min, and the pellet was mixed with 1 mL sterile distilled water. A five-step, 10-fold serial dilution was performed, and 5 µL from each dilution was spotted onto YPD (standard and glucose-restricted) plates. Plates were incubated at 30 °C for 2 days before assessing growth. For each sample, four replicate plates were prepared.

mtDNA mutation rate analysis

The frequency of mutants resistant to antibiotics like erythromycin, chloramphenicol, oligomycin, and antimycin, which affect different mitochondrial functions, can be used to estimate mitochondrial point mutagenesis levels. Erythromycin resistance results from mutations at various sites in the mitochondrial 21S rRNA gene (Kaniak-Golik and Skoneczna, 2015). First, standard and calorie-restricted liquid YPG media were inoculated with a single colony of the mutant yeast strain. Following two days of incubation, the cultures were adjusted to an OD_{600} of 0.4 using sterile distilled water. Subsequently, a second dilution was performed, and 30 µL of each diluted culture was plated onto solid standard and calorie-restricted YPG plates containing erythromycin (1 mg/mL). The plates were incubated at 30 °C for 21 days to monitor the formation of colony-forming units (CFUs). The presence of CFUs indicated erythromycin resistance resulting from mutations. Mutation rates (µ) were calculated as $\mu = -\text{In (Po)}/n \times \text{In2}$, where "n" is the total number of cultures and "Po" is the percentage of cultures free of erythromycin-resistant mutations. Lastly, differences between groups were assessed using a single-factor

ANOVA.CFU measurements were carried out in three replicates.

mtDNA abundance and distribution

The *gcn5Δ* yeast strain was cultured in liquid YPD (standard and CR) media for 25 days, because when yeast cells grow on glucose, their mitochondrial DNA (mtDNA) begins to proliferate in copy number at the start of the diauxic shift and continues to rise throughout the respiration phase (Galeota-sprung, Fernandez and Sniegowski, 2021) and the budding yeast has been observed to enter a stationary phase following an incubation period of 20 to 25 days (Sudiyani et al., 2021). After 25 days, yeast cells were mixed with 100% ethanol at a 1:3 ratio in a 1.5 mL microfuge tube and incubated at room temperature for 30–60 minutes. The mixture was then centrifuged at 2,500 rpm for 1 minute, the supernatant was discarded, and the pellet was resuspended in 1 mL of 1× PBS. After a second centrifugation under the same conditions, the supernatant was discarded, and the pellet was resuspended in 200 µL of 1× PBS containing DAPI at a 1:2000 dilution (prepared by adding 0.5 µL of a 2.5 mg/mL DAPI stock solution to 1 mL PBS). After 30 minutes of incubation in the dark at room temperature, cells were centrifuged, and the supernatant was discarded. A drop of the resulting cell pellet was then mounted on a microscope slide and observed under a fluorescence microscope. The same procedure was done twice. Corrected Total Cell Fluorescence (CTCF) was quantified using ImageJ software. For each slide, three measurements were taken from three different views, each containing the same number of cells. A graph was created from the mean CTCF value to compare the fluorescence level. CTCF was calculated using the formula: CTCF = Integrated Density - (Area of selected cell × Mean fluorescence of background readings). Statistical differences between fluorescence measurements were analyzed with a single-factor ANOVA.

Results and discussion

Deletion of GCN5 does not abolish the lifespan-extending effect of calorie restriction in yeast

The length of time that non-dividing cells may live is known as the chronological lifespan (CLS). CLS assays are generally conducted by maintaining cells in the stationary phase for extended periods (Chadwick *et al.*, 2016).

In this experiment, we analyzed the chronological lifespan of the *gcn5Δ* yeast strain under standard and calorie-restricted conditions for 30 days to investigate how calorie restriction affects cellular viability in the absence of *GCN5* during the non-dividing phase. The mean chronological lifespan of the *gcn5* null mutant cells was 16.80 days under standard conditions and 17.56 days under calorie-restricted (CR) conditions, representing an approximate 4.52% increase. Therefore, yeast cells cultured in CR media exhibited a significantly longer ($P < 0.05$) lifespan compared to those grown in standard YPD media **(Figure 1A)**.

Yeast cells often have longer lifespans due to the retrograde reaction. But the retrograde response's ability to lengthen life is inhibited when *GCN5* is deleted (Mutlu and Puigserver, 2021). Again, *GCN5* facilitates CLS extension by enabling redox homeostasis and stress tolerance. The ability of early-stationary phase *gcn5Δ* cells to withstand exogenous oxidative stress was severely compromised (K. Li *et al.*, 2023) which may lead to a shortened lifespan.

External factors may affect longevity, which is not dependent on *GCN5* gene. Exposure of *S. cerevisiae* to acetic acid, a cell-extrinsic mediator of cell death during chronological aging, results in cell death, whereas dietary restriction extends lifespan by lowering or removing extracellular acetic acid (Ludovico *et al.*, 2001; Burtner, Murakami and Kennedy, 2010). Interestingly, under low glucose conditions, without Gcn5, Set2 protein stability persists even in pro-aging circumstances, resulting in sustained expression of genes related to aging, thus prolonging yeast lifespans (Y. Li *et al.*, 2023). Calorie restriction-induced longevity also relies on transcription factors downstream of Ras/PKA, TOR, and Sch9 (Wei *et al.*, 2008), which are not strictly dependent on *Gcn5* HAT activity. Together, PKA, TOR, and Sch9 oversee ribosome biogenesis, cellular metabolism, and the stress response (Tsang and Lin, 2016), as well as autophagy (Conrad *et al.*, 2014), thereby enhancing lifespan positively. Therefore, it can be said that calorie restriction improves the chronological lifespan of *gcn5Δ* yeast mutants, suggesting that lifespan extension does not strictly require *GCN5*.

CR reduces the growth rate of gcn5Δ cells but extends lifespan independently of GCN5

Optical density (OD) measurements were used to monitor the growth dynamics of *gcn5Δ* cells for 30 days under both standard (S) and calorie-restricted (CR) settings. The *gcn5Δ* cells maintained a considerably higher cell density in standard media, displaying a higher optical density (~1.1-1.3) during the course of the culture time. In contrast, cells cultured under calorie restriction showed persistently lower optical densities (~0.5-0.9) **(Figure 1B)**, suggesting slower growth. Although the OD of mutant cells in CR conditions gradually stabilized in the later days of culture.

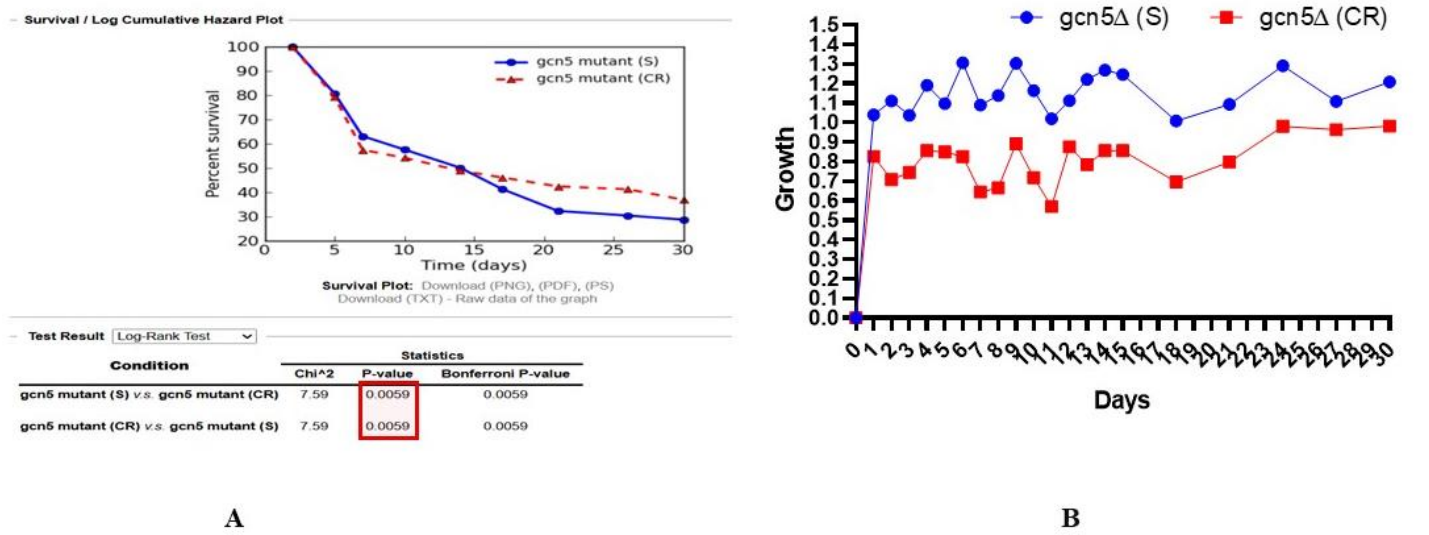


Figure 1. Chronological lifespan (CLS) and growth dynamics of *gcn5Δ* cells under standard (S) and calorie-restricted (CR) conditions: (A) CLS assay shows extended survival under CR (*P < 0.05), indicating *GCN5*-independent longevity; (B) Growth curves reveal slower growth of *gcn5Δ* cells under CR, with OD stabilizing at later time points.

Cells deficient in *GCN5* utilized glucose similarly to wild-type cells (Shivarathri *et al.*, 2019). Yeast favors fermentation as the primary method of producing ATP when a high quantity of sugar is present. It's interesting to note that the particular growth rate is maintained at its highest level during the fermentation process (Olivares-marin *et al.*, 2018). *S. cerevisiae* exhibited higher growth in cultures with 2% glucose, whereas growth was reduced at 0.5% (Ramos-gomez *et al.*, 2017). Another study found that yeast uses mitochondrial respiration to create ATP when fermentable carbon levels are low, which keeps growth rates slower (Olivares-marin *et al.*, 2018). Slower growth is usually brought on by the depletion of external carbon and energy supplies during the non-dividing period (Bisschops *et al.*, 2017). In this phase, quiescent cells of eukaryotic microorganisms can survive for long durations without any nutrients (Gray *et al.*, 2004; Kumar and Srivastava, 2016). Additionally, nutrient scarcity regulates cell cycle progression, leading to cell arrest in the G1 phase and the transition into the resting G0 phase (Pedruzzi *et al.*, 2003; Wanke, Pedruzzi and Virgilio, 2005). A study found that lifespan extension occurs when the progression of the cell cycle is halted or slowed (Longo, Mitteldorf and Skulachev, 2005).

gcn5Δ cells show improved respiration under CR

Cells with impaired mitochondria are unable to use respiratory substrates like ethanol or glycerol. Therefore, we assessed the impact of calorie restriction on the respiratory efficiency of *gcn5*Δ cells by analyzing their growth in both standard and calorie-restricted respiratory media. Results suggested that *gcn5*Δ cells exhibited slow growth on standard YPG media, but had enhanced growth on YPG under calorie restriction **(Figure 2A)**, indicating that CR enhances their respiratory efficiency. Besides, the growth of cells in glucose (both YPD and YPD CR) was similar, as they were found by a growth curve analysis in liquid YPD and YPD CR media **(Figure 2B)**.

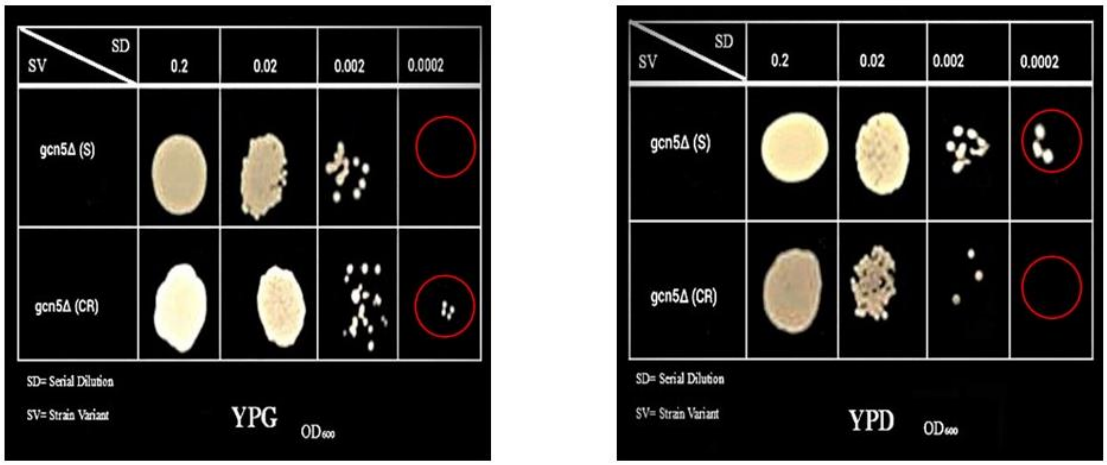


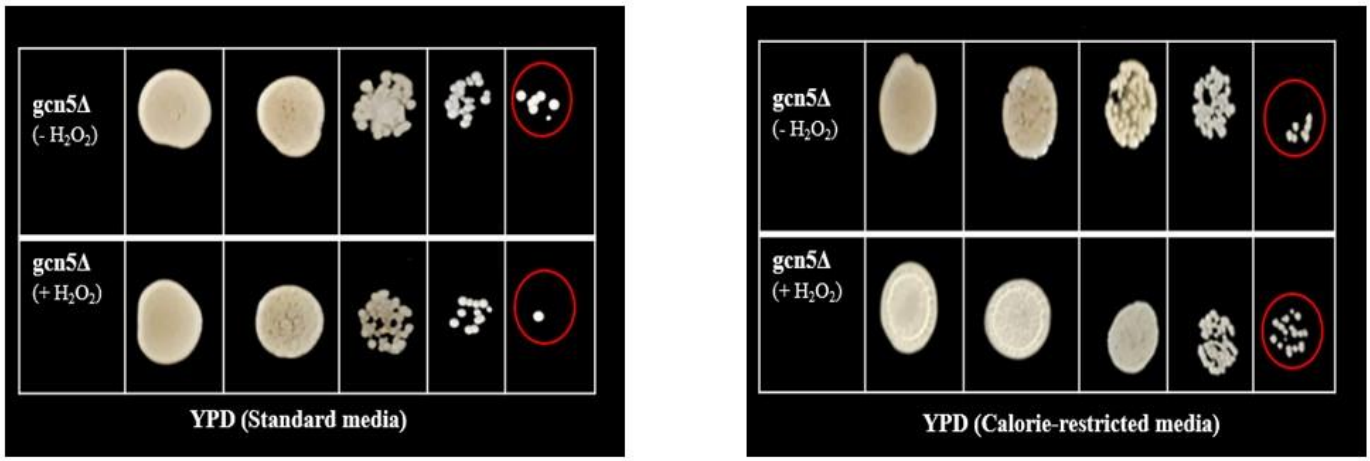
Figure 2. Respiratory status of the *gcn5Δ* cells: A) Cells showed reduced growth on standard YPG, whereas CR significantly improved growth, indicating enhanced mitochondrial respiratory efficiency; B) Growth in glucose-containing media (YPD and YPD CR) was comparable, as shown by liquid culture growth curves.

GCN5 is essential for growth under respiratory conditions and for oxygen usage in budding yeast (Canzonetta *et al.*, 2016) and the *gcn5Δ* mutants were previously demonstrated to exhibit defects in respiratory growth (K. Li *et al.*, 2023). In yeast mitochondria, the Gcn5 protein exists within the mitoplasts and is situated in the inner mitochondrial membrane. Its removal impacts the mitochondrial characteristics and is associated with a significant reduction in mitochondrial DNA levels (Montanari et al., 2019). These are consistent with compromised respiratory capacity, further explaining poor YPG growth.

CR enhances the rate and effectiveness of utilizing respiratory substrates like glycerol (Tahara *et al.*, 2013). Under CR, yeast transitions from fermentation to respiration. Respiration stimulates specific conserved longevity factors and is linked to extensive physiological alterations that enhance survival (Skinner and Lin, 2010). Additionally, CR minimizes the excess generation of ROS and oxidative injury, resulting in improved mitochondrial performance (Bhatti *et al.*, 2018). In summary, these findings suggest that calorie restriction may partially offset the loss of *GCN5* by enhancing respiratory efficiency, mitochondrial function, and the growth of *gcn5*Δ cells in non-fermentable conditions.

Oxidative stress resistance under CR is GCN5-independent

We examined colony survival on standard and calorie-restricted YPD medium with or without hydrogen peroxide (H_2O_2) treatment to ascertain if calorie restriction (CR) affects the *gcn5Δ* mutant's sensitivity to oxidative stress. After being exposed to H_2O_2 in normal YPD, the cells showed significantly decreased growth, with only a few colonies visible at the highest dilution, demonstrating great oxidative stress sensitivity **(Figure 3A)**. On the other hand, mutant cells showed significantly enhanced survival under calorie-restricted conditions, maintaining strong colony formation even in the presence of H_2O_2 **(Figure 3B)**. These findings imply that calorie restriction (CR) stimulates *GCN5*-independent protective mechanisms that improve cellular resistance to H_2O_2.



(A) **(B)**

Figure 3. Evaluation of oxidative stress tolerance: A) *gcn5Δ* cells show oxidative stress sensitivity on standard YPD media; B) CR significantly improves oxidative stress resistance in *gcn5Δ* cells.

GCN5 is necessary for several stress responses in yeast. Stress sensitivity is caused by abnormalities in the conserved histone acetyltransferase domain of *GCN5* (Xue-franzén *et al.*, 2010). Another study by Gaupel et al also suggested that the cellular response to oxidative stress is modulated by *GCN5*. The *gcn5Δ* deletion mutant exhibits increased cell death and reactive oxygen species accumulation (Gaupel, Begley and Tenniswood, 2015).

The mitochondrial electron transport chain (ETC) was enhanced by CR on both a transcriptional and translational level. In fact, CR raised mitochondrial membrane potential (MMP) and reduced the overall production of reactive oxygen species (ROS) (Choi, Choi and Lee, 2011). Again, according to current research, hydrogen peroxide (H_2O_2) and other ROS serve as crucial secondary messengers in the control of several physiological processes in mammals (William and Michael, 2007). For instance, pro-survival signaling pathways regulated by p53, NF-κB, AP-1, and other molecules are activated by H_2O_2 (Groeger, Quiney and Cotter, 2009). Under calorie restriction, a small and controlled increase in H_2O_2 activates protective enzymes superoxide dismutase, which reduce more harmful ROS, leading to longer lifespan (Mesquita *et al.*, 2010). Furthermore, another study found that CR induces H_2O_2 resistance via a mechanism independent of respiration (Toledano, Labarre and Nystro, 2011).

CR reduces mtDNA mutation rate in the absence of GCN5

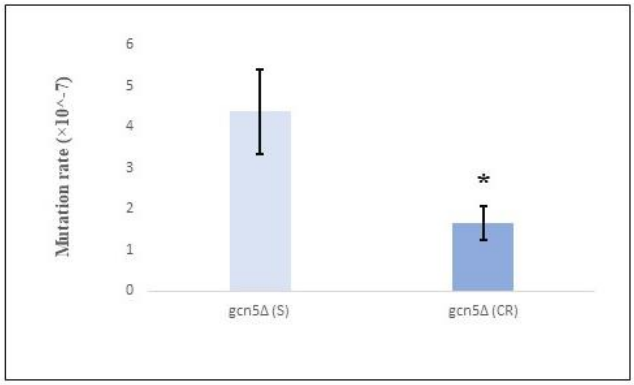
In comparison to calorie restriction, the *gcn5Δ* strain's mutation rate was noticeably greater under normal growth media. The mutation rate under normal conditions was around $4.4\times10-7$ (mean ± SD: $4.4\pm1.1\times10-7$). Conversely, the *gcn5Δ* strain under calorie restriction had a mutation rate of around $1.7\times10-7$ (mean ± SD: $1.7\pm0.4\times10-7$) **(Figure 4A)**. In comparison to the standard condition, the calorie-restricted strain's mutation rate has decreased by more than 2.5 times ($p<0.05$). According to this research, calorie restriction is linked to a lower rate of spontaneous mutation even when there is no functioning *GCN5*.

There is mitochondrial DNA (mtDNA) instability in the *gcn5Δ* mutant strain (Cirigliano *et al.*, 2025). Moreover, high-sugar diets increase the amount of glucose accessible to mitochondria, which increases the amount of substrate available for ATP production. As a result, the mitochondria are overactive and generate more ROS (Hurrle and Hsu, 2017). Furthermore, according to the oxidative damage attenuation theory, high nutrient/calorie intake increases metabolism, which raises ROS levels (Ray, Huang and Tsuji, 2012). ROS is thought to be a major contributor to mtDNA mutations and can produce oxidative base lesions in mtDNA (Shokolenko *et al.*, 2009).

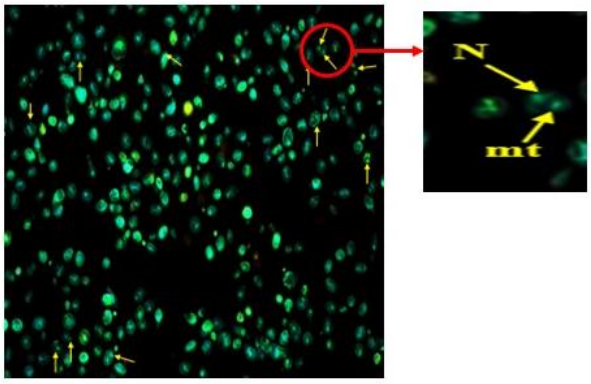
A well-known experimental technique called caloric restriction (CR) lengthens the maximum life span of several animals and lowers the pace at which mtDNA mutations accumulate (Gredilla, 2011). Mechanistically, CR reduces the production of mitochondrial reactive oxygen species (ROS) (mitROS) and oxidative damage to mtDNA and increases maximum longevity (Sanz et al., 2006). In addition to reducing oxidative stress, CR also stimulates mitophagy, which makes it easier to eliminate damaged mitochondria (Mehrabani *et al.*, 2020). Together, these results imply that CR may reduce oxidative damage and the long-term buildup of mitochondrial DNA mutations, therefore slowing down the pace of aging (Barja, 2004).

Mitochondrial DNA abundance is increased by CR irrespective of GCN5

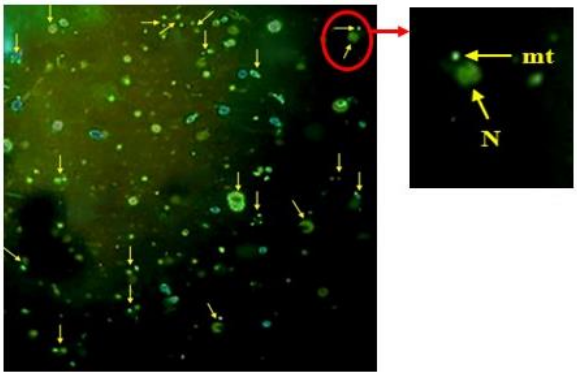
DAPI-stained *gcn5Δ* cells from both culture media were observed under a fluorescence microscope at 40X magnification. A prominent round structure was identified as the nucleus (N), whereas the smaller, scattered ones corresponded to mitochondrial DNA (mtDNA). Mutant cells cultured in calorie-restricted media displayed enhanced mtDNA abundance, as observed by microscopy **(Figure 4B, 4C**). Quantitative assessment using ImageJ software further supported this observation. Analysis of the mean corrected total cell fluorescence (CTCF) values demonstrated that the mean fluorescence intensity in calorie-restricted (CR) cells was approximately 1.7-fold higher **(Figure 4D)**, reflecting a significant increase in overall mtDNA abundance ($P < 0.05$).



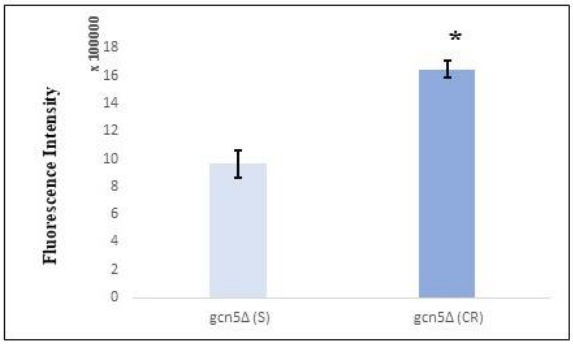
4A



4B (Standard media)



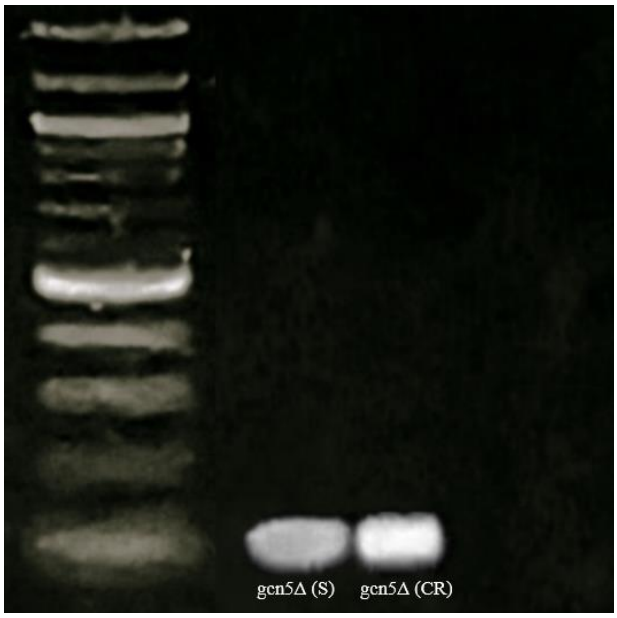
4C (Calorie-restricted media)



4D

Figure 4. mtDNA mutation rate and abundance: A) mtDNA mutation rate was more than 2.5-fold (*p <0.05) lower under CR, reflecting improved genome stability; B and C) Cells cultured under calorie-restricted (CR) conditions showed increased mtDNA abundance compared to standard (S) media (mt: mitochondrial DNA and N: nucleus); D) Mean corrected total cell fluorescence (CTCF) in CR cells was ~1.7-fold higher, indicating a significant increase in mtDNA content (*P < 0.05).

To further evaluate mitochondrial DNA, the *COX2* gene was amplified using PCR. A visual comparison of the PCR results showed that mutant cells grown in a glucose-restricted environment had greater quantities of mtDNA. This technique offers a qualitative indicator of relative mtDNA abundance because no internal reference gene was studied **(S. Figure 1)**.



Supplementary Figure 1. Agarose gel electrophoresis of PCR products of the mitochondrial *COX2* gene: Lane 1 contains the DNA ladder. Both standard (lane 2) and calorie-restricted (lane 3) cells produced ~200 bp *COX2* PCR products. Qualitative analysis indicates a relatively higher mitochondrial DNA abundance in glucose-restricted mutant cells compared to controls.

In high glucose media, ROS are produced as a result of an inefficient electron transport chain in the mitochondria. As a result, mitochondrial DNA, which codes for mitochondrial proteins, has poor transcription and protein synthesis, which ultimately leads to mitochondrial malfunction (Zhang *et al.*, 2023). In mammals, *GCN5L1* plays a crucial role in controlling mitochondrial content through the synchronized regulation of mitochondrial biogenesis and mitophagy (Scott *et al.*, 2014). These likely explain why the mutant cells cultured in standard glucose media exhibited lower mitochondrial content.

Under calorie-restricted conditions, mitochondria produce fewer reactive oxygen species, use less oxygen, and have lower membrane potentials. Surprisingly, however, they are still able to produce the essential amount of ATP. Restricting calories encourages the growth of mitochondria while also lowering oxidative stress (Hunt *et al.*, 2006). CR also improves mitochondrial function through enhanced mitochondrial fusion and biogenesis (Pintus, Floris and Rufini, 2012). Mechanistically, Caloric restriction (CR) causes PGC-1α to become active in the nucleus, which then triggers the transcription of genes essential for mitochondrial biogenesis and function (Scholle et al., 2020). Glucose depletion also induces ethanol-driven respiration and enriches genes associated with mitochondrial function and gluconeogenesis (Repressor, 2014). Additionally, calorie restriction triggers autophagy, and longer lifespans may result, at least partially, from improved elimination of oxidatively damaged mitochondria and their altered mtDNA (Kim, Rodriguez-enriquez and Lemasters, 2009). Consistent with this, CR promotes an increase in the amount of mitochondria (Cozzi *et al.*, 2005; Civitarese *et al.*, 2007)

Conclusion

In conclusion, this study offers compelling evidence that the lifespan-extending and metabolic advantages of Calorie Restriction (CR) in *Saccharomyces cerevisiae* are mostly mediated by processes that do not depend on the histone acetyltransferase *GCN5*. Although *GCN5* is an essential integrator of nutrient signaling, the fact that the *gcn5Δ* under CR showed a marked increase in Chronological Lifespan (CLS) calls into question whether this chromatin-modifying factor is required as an obligatory upstream regulator of all CR-mediated longevity pathways. Longevity was improved by the combined effects of glucose decrease on growth dynamics, respiratory efficiency, antioxidant activity, and mitochondrial DNA stability. Future studies must aim to identify the specific signaling pathways that are responsible for the *GCN5*-independent effects of calorie restriction. A robust understanding of these pathways will be fundamental for developing pharmacological interventions that specifically mimic the pro-longevity effects of caloric restriction, while reducing off-target impacts from global transcriptional modulation.

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Declaration of interests

The authors have stated that there are no conflicting interests.

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