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ORIGINAL PAPER

Chronological lifespan regulation of *Saccharomyces cerevisiae* by leucine biosynthesis pathway genes via *TOR1* and *COX2* expression regulation

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Abstract

Backgrounds: Leucine is involved in various cellular mechanisms, including protein metabolism, insulin signaling, and longevity control. Nevertheless, the contribution of leucine metabolism genes to longevity have not been thoroughly studied.
Methods: Several mutants of leucine biosynthesis genes were constructed, and their effect on yeast lifespan and various phenotypes were examined.
Results: Several deletion mutants increase yeast lifespan. Among those, *LEU2* and *leu4*Δ cells exhibit significantly increased lifespan, moderately increased reactive oxygen species (ROS) generation, decreased Tor1p expression, significantly increased expression of a cytochrome c oxidase subunit, and decreased cell death. In these cells, reduced Tor1p seemed to stimulate a slight increase in ROS generation which stimulates Cox2p expression that can prevent cellular damage. Indeed, the rate of cell death of *LEU2* and *leu4*Δ cells was drastically decreased.
Conclusion: *LEU2* and *LEU4* seem critical in determining yeast lifespan by providing a hormetic effect that promotes yeast longevity.

Keywords: Yeast, Leucine, *LEU2*, *LEU4*, Chronological lifespan

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Introduction

Lifespan is associated with many factors, including longevity genes, nutrition, and other environmental factors. One of the most well studied factors is caloric restriction (CR) which increases the health and lifespan of a wide range of species[1]. Extensive studies on CR-induced longevity have revealed connections among target of rapamycin (TOR) signaling, mitochondrial respiration, ROS generation, and many other cellular mechanisms. CR-induced reduction of TOR activity is known to reduce cellular ROS levels and increase the lifespan of many species, including yeast, mice, and human cells[1-3]. Yeast grown in CR conditions exhibits significantly increased chronological lifespan (CLS), decreased Tor1p, and increased Cox2p expression[4]. Furthermore, carbohydrates, lipids and proteins have also been shown to affect aging. In addition to functioning as energy sources and cellular building blocks, supplementation or depletion of some amino acids (AAs), such as methionine and branched-chain amino acids (BCAA), modulate the health and lifespan of many species[5,6]. BCAA supplementation was shown to promote yeast CLS[7]. In addition, BCAA levels were up-regulated in long-lived *C. elegans*[8], and BCAA-supplemented mice exhibited prolonged survival[5]. Recently, BCAA was suggested as a conserved physiological aging regulator of various species from nematodes to mice[8].

Among the BCAA, leucine is involved in various cellular mechanisms. It serves as a building block for proteins, as a cellular energy source by catabolism through the TCA cycle in mitochondria, and as an intracellular signaling molecule. Leucine is also known to regulate protein synthesis, protein homeostasis, glu-

cose tolerance, and insulin signaling[5,9-12]. Furthermore, leucine stimulates human muscle protein synthesis through mammalian target of rapamycin (mTOR) activation[12,13]. This suggests that leucine metabolism is closely related to sarcopenia. Moreover, leucine supplementation promotes the chronological longevity of yeast and other species[7,8,14]. More evidence showing the role of cellular leucine levels and leucine biosynthesis-related genes was derived from the extended lifespan of yeast in the presence of *LEU2*, which is mutated in most laboratory yeast strains[7,14].

Leucine is the most abundant AA in many dietary proteins[11]. While leucine is a required AA that must be supplemented because it is not synthesized in human or other higher eukaryotic cells, it can be synthesized through a leucine biosynthesis pathway (LBP) in yeast[15]. The first committed step of leucine synthesis is the production of α-isopropylmalate (α-IPM) from α-ketoisovalerate (α-KIV) and acetyl-CoA by the products of *LEU4* and *LEU9* which encode α-IPM synthase[15-17]. α-IPM is not just an intermediate in the LBP. More importantly, α-IPM functions in transcriptional regulation with Leu3p, a dual transcription regulator that functions as an activator in the presence of α-IPM and as a repressor in the absence of α-IPM. *LEU3*-encoded protein does not directly function in the LBP, but Leu3p controls the expression of most genes in the LBP[15]. Deletion of *LEU3* increased yeast CLS[7], suggesting that regulation of the expression of LBP genes affects yeast longevity.

Regardless of the importance of leucine in longevity, the genes involved in its metabolism have not been thoroughly studied for their contribution to longevity control. In this study, we analyzed several yeast mutants in genes of the LBP to study their functions in yeast CLS regulation. Our results show the importance of *LEU2* and *LEU4* genes in yeast CLS determination. We further elucidate the mechanisms of their effects on yeast lifespan.

Materials & Methods

Yeast strains

EMY73 (MATa *his3*-Δ1, *leu2*-3, -112, *ura3*-52, *trp1*Δ::*URA3*) and its isogenic strains were used in this study (Table 1). To generate *LEU2* cells, *LEU2* ORF was introduced to the genome of EMY73 and EMY74.7 by transforming the DNA fragment that contains *LEU2* ORF and its flanking region. *LEU3, LEU4, LEU9, BAT1,* and *BAT2* genes were deleted by replacing the entire open reading frame (ORF) of the genes with the *URA3* gene. Deletion of the gene was verified by PCR using a pair of oligonucleotides flanking each ORF.

Table 1. Yeast strains used in this study.

Strains	Genotype	Source
EMY73	MATa *his3*-Δ1 *leu2*-3,-112 *ura3*-52 *trp1*Δ::*URA3*	Reference 27
EMY74.7	MATa *his3*-Δ1 *leu2*-3,-112 *ura3*-52 *trp1*Δ	Reference 27
YSL956	EMY73 *LEU2*	This study
YSL961	EMY74.7 *LEU2*	This study
YSL962	YSL961 *leu4*Δ::*URA3*	This study
YSL969	YSL961 *Bat2*Δ::*URA3*	This study
YSL970	YSL961 *leu4*Δ::*URA3*	This study
YSL971	YSL961 *leu3*Δ::*URA3*	This study
YSL975	YSL961 *leu9*Δ::*URA3*	This study
YSL979	YSL961 *bat1*Δ::*URA3*	This study

Determination of cell growth

All yeast cells used in this study were grown in synthetic complete (SC) medium containing nitrogen base, ammonium sulfate, and 12 amino acids at 30°C[4] unless otherwise specified. To examine the effect of *leu2* and *leu4* mutation on growth, yeast cells were grown to the stationary stage in SC medium. The cells were diluted in dH_2O and then plated on SC plates (+ leucine in Figure 1B) or on leucine-depleted synthetic medium plates (− leucine in Figure 1B). The plates were incubated at 30°C for 2 days before images were captured.

CLS Assay

CLS was analyzed as previously described[4]. Briefly, yeast was grown in SC medium to the stationary stage and diluted in fresh SC medium to the OD_{600} of 0.03. Once the diluted cells were grown to the stationary stage at 30°C, yeast cells were harvested, washed twice with dH_2O, and then resuspended in dH_2O. Then, the cells were kept in dH_2O, and aliquots of cells were taken and plated on YPD (1% yeast extract, 2% peptone, 2% glucose) medium at the indicated time points in Figure 1. The plates were incubated at 30 °C for 3-5 days, and the number of colonies on each plate was counted to determine the portion of cells that maintain proliferative ability. Survival curves represent an average of at least three independent experiments performed in duplicate.

Vital dye exclusion assay

Yeast cells were grown as in the CLS assay and harvested at the indicated time points in Figure 2. The cells were washed, resuspended in phosphate buff-

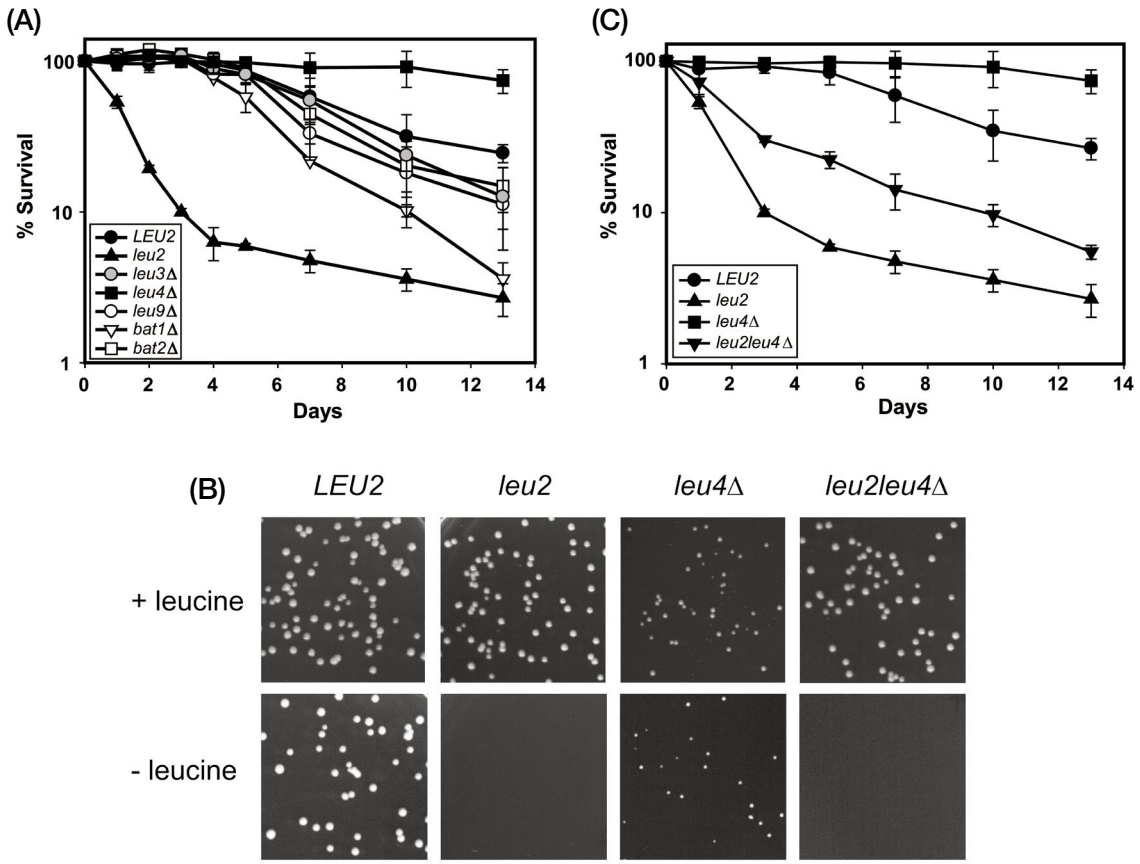

Figure 1. CLS and growth of *LEU2* and deletion mutants of leucine biosynthesis genes. (A) CLS of leucine biosynthesis gene deletion mutants. All tested mutants exhibited longer CLS than *leu2* cells. However, only *leu4*Δ mutant displayed further extended CLS than *LEU2* cells. (B) All four tested strains grow well in the presence of leucine supplementation. Both *leu2* and *leu2leu4*Δ cells, which do not have *LEU2*, are unable to grow in the absence of exogenous leucine supplementation (−leucine). Growth of *leu4*Δ cells is retarded, both in the absence and presence of exogenous leucine supplementation. (C) *LEU4* deletion extends CLS of both *LEU2* and *leu2* cells. CLS of *leu4*Δ and *leu2leu4*Δ cells is extended compared to that of *LEU2* and *leu2* cells, respectively.

ered saline (PBS), incubated with 0.1% Evans blue dye (Sigma, St. Louis, MO, U.S.A.) for 15 min, and rinsed once with PBS. Micrographs were captured using a ZEISS Axioplan2 microscope (ZEISS, Germany) featuring ×400 and ×1000 objectives and equipped with a Dage-MTI DC-330 color camera (DAGE-MTI, USA). For analysis of dead cell percentage, at least 1,000 cells per strain from three independent experiments were evaluated.

Measurement of ROS formation

Yeast cells were grown as in the CLS assay. Aliquots of cells were harvested and treated with 2.5 mg/mL dihydrorhodamine 123 (DHR123, Sigma, St. Louis, MO, U.S.A.) for 1 h. The cells were then washed twice with 1× PBS before fluorescence-activated cell sorting (FACS) analysis (Becton-Dickinson, Franklin Lakes, NJ, U.S.A) to verify ROS formation. At each indicated time point, aliquots of each culture were taken and the ROS levels of 20,000 cells was analyzed using a Becton Dickinson FACSCalibur (BD Biosciences, San Jose, CA, USA). Cell Quest software was used for analysis.

Western blot analysis

Cox2p and Tor1p levels were analyzed by Western blot analysis as previously described4. Briefly, cells were grown in SC medium and whole extracts were prepared. The proteins were separated on 10% SDS-PAGE, and transferred to PVDF membranes (Millipore, USA) using a wet transfer apparatus (Invitrogen, USA). Tor1p, Cox2p, and actin were detected using anti-Tor1 antibody (Santa Cruz Biotech, Santa Cruz, CA, U.S.A.), anti-Cox2 antibody (Invitrogen, Grand Island, NY, U.S.A.), and anti-actin antibody (Abcam, Cambridge, MA, U.S.A.), respectively. The autoradio-

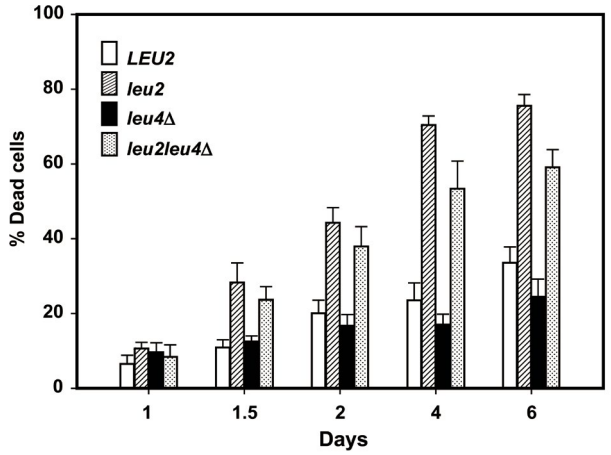

Figure 2. Cell death is decreased in *LEU2* and *leu4*Δ cells. Cell death of *LEU2* and *leu4*Δ cells is significantly lower than that of *leu2* and *leu2leu4*Δ cells. These results indicate that leucine biosynthesis or sufficient cellular leucine is required to maintain yeast CLS. Each bar represents the mean with standard deviation of 3 or more experiments.

graph images were scanned and quantified by densitometry using the ImageJ software (https://imagej.nih.gov/ij/download.html).

Results

Influence of leucine biosynthesis genes on CLS

The most commonly used laboratory *S. cerevisiae* strains are leucine biosynthesis incompetent due to mutations in *LEU2* for experimental manipulation. Consequently, the majority of aging studies in yeast have been conducted in the absence of intact *LEU2*[4,14,18-20]. Because we wanted to examine the effect of LBP genes on yeast longevity, we first introduced *LEU2* into the genome of EMY73, a laboratory wild-type strain containing *leu2-3,-112* mutations (Table 1). Then, deletion mutants of several leucine biosynthesis related genes, *BAT1*, *BAT2*, *LEU3*, *LEU4* and *LEU9*, were constructed in the background of *LEU2* (Table 1). Although Leu4p and Leu9p participate in the same process, Leu4p is the main isozyme, accounting for more than 80% of the total α-IPM synthase activity[15], which processes the first step in leucine biosynthesis by converting α-KIV to α-IPM. *LEU2* encodes β-isopropylmalate (β-IPM) dehydrogenase which catalyzes the third step in the LBP. On the other hand, *BAT1* and *BAT2* encode BCAA transaminases that function in several different steps of the BCAA biosynthesis pathway, including the last step of leucine biosynthesis to convert the last intermediate, α-ketoisocaproate (KIC), to leucine[15].

To investigate the functions of genes in the LBP in yeast longevity regulation, the CLS of the constructed mutants was analyzed. As shown in Figure 1, *LEU2* cells exhibited an extended CLS compared to EMY73 (referred as *leu2* cells hereafter). The CSL increase of *LEU2* cells was expected since the presence of *LEU2* has been reported to extend the CLS of yeast grown in minimal synthetic medium[7], suggesting that maintaining leucine levels by leucine biosynthesis is important for yeast CLS. All the tested mutants, *bat1*Δ, *bat2*Δ, *leu3*Δ, *leu4*Δ, and *leu9*Δ, exhibited extended CLS compared to *leu2* cells. However, only the *leu4*Δ mutant displayed extended CLS compared to *LEU2* cells (Figure 1A), indicating that *LEU2* and *LEU4* are key genes modulating CLS through leucine metabolism. The absence of CLS extension in *leu3*Δ cells was unexpected because a previous study showed a dramatically increased CLS in *leu3*Δ cells in the absence of AA supplementation[7]. This discrepancy might be due to differences in the genotypes of the *leu3*Δ strains. The *leu3*Δ cells in this study have an intact *LEU2* while the *leu3*Δ strain in the previous study[7] had a *leu2*Δ*0* mutation. Indeed, the *leu3*Δ cells in our study also exhibited a substantially extended CLS compared to that of *leu2* cells (Figure 1A).

Slow growth of *leu4*Δ mutant is not the result of cellular leucine depletion

The significantly increased CLS of *LEU2* compared to *leu2* cells is thought to be the result of increased leucine biosynthesis, indicating the importance of *LEU2* and normal leucine biosynthesis in yeast lifespan determination. With abolished Leu2p function, *leu2* cells exhibited growth defects in the absence of exogenous leucine supplementation (Figure 1B) and a decreased CLS compared to that of *LEU2* cells (Figure 1A). On the other hand, during leucine biosynthesis, Leu4p is responsible for the majority of α-IPM production[15]. A small portion of α-IPM is also produced by the product of *LEU9*, which is a paralog of *LEU4*[17]. α-IPM is converted to β-IPM by Leu1p, and then Leu2p converts β-IPM to KIC, which is the immediate precursor of leucine. Moreover, α-IPM functions as a transcription regulator along with Leu3p which regulates each of LBP genes. Leu3p-α-IPM complex activates six genes out of nine genes involved in leucine biosynthesis. In the absence of α-IPM, Leu2p activity is down-regulated[15]. Thus, disruption of *LEU4* would decrease cellular α-IPM levels and possibly the amount of cellular leucine biosynthesis. However, leucine accumulation in *leu4*Δ cells was previously reported[21], suggesting that the increased CLS of *leu4*Δ cells is caused by mecha-

nisms other than the reduction of leucine biosynthesis.

To verify the role of *LEU4* in the regulation of CLS, we first verified the effect of *leu4*Δ on yeast growth. *leu4*Δ cells formed colonies well but grew slower than *LEU2* cells in the absence of leucine supplementation. Interestingly, the growth of *leu4*Δ cells in the presence of exogenous leucine supplementation was also distinctly slower than both *LEU2* and *leu2* cells (Figure 1B), suggesting that the slow growth of *leu4*Δ cells is not entirely the result of impaired leucine biosynthesis. Slow growth of *leu4*Δ mutant was also observed in other studies, in which it was demonstrated that the slow growth of these cells was not due to leucine depletion[21]. Taken together, these results suggest that sufficient leucine is produced in *leu4*Δ cells, and that the slow growth of *leu4*Δ mutant is not the result of leucine depletion.

In fact, the *leu2leu4*Δ mutant was not able to grow in the absence of leucine supplementation (Figure 1B). However, the growth of the *leu2leu4*Δ strain in the presence of exogenous leucine supplements is less retarded than that of the *leu4*Δ mutant. These results suggest that the slow growth of *leu4*Δ cells could be the result of an impairment in an undefined Leu4p function. In addition, *leu4*Δ increases CLS in both the presence and absence of *LEU2*. *leu2leu4*Δ cells also exhibited an extended CLS compared to *leu2* cells (Figure 1C). The extended CLS of *leu2leu4*Δ cells and its growth incompetence in the absence of exogenous leucine supplementation also suggest that *leu4*Δ contributes to yeast longevity via other mechanisms beyond reducing the concentration of an intermediate in the LBP.

*leu4*Δ increases cell viability

CLS is related to the rate of cell death. While leucine supplementation extends CLS, leucine starvation induces cell death of yeast and human cancer cells[22,23]. When *LEU2* function is impaired in yeast, leucine biosynthesis is abolished and cells are starved for leucine, such that *leu2* cells are unable to grow in the absence of exogenous leucine supplementation (Figure 1B). Thus, the rates of cell death in *leu2* and *leu2leu4*Δ cells are predicted to be elevated due to leucine depletion. To verify *LEU2* and *LEU4* influence on yeast cell death, the rate of cell death of *LEU2*, *leu2*, *leu4*Δ, and *leu2leu4*Δ cells was examined during CLS assays. As predicted from the results in Figure 1, cell death was pronounced in the absence of *LEU2*. On the other hand, the proportion of dead cells in both the *LEU2* and *leu2* strains was decreased by *leu4*Δ (Figure 2). These results correspond to the extended CLS of *leu4*Δ and *leu2leu4*Δ cells compared to *LEU2* and *leu2* cells, respectively.

*leu4*Δ modulates Tor1p levels

Extended lifespan is closely related to reduced-TOR signaling in various organisms[18]. Lifespan extension by reduced TOR signaling is due to modulation of many cellular processes in response to nutrients, especially AA. In addition, leucine is a signaling molecule that regulates mTOR[24]. Also, *tor1*Δ is known to significantly reduce the rate of cell death in leucine-starved cells[22]. Thus, the extended CLS of *LEU2* and *leu4*Δ cells (Figure 1A, 1B) might be caused by Tor1p modulation. To verify the relationship between CLS modulation by *LEU2*/*LEU4* and the TOR pathway, Tor1p expression was analyzed. Tor1p levels in *LEU2* cells were significantly lower than in *leu2* cells. Furthermore, the amount of Tor1p in *leu4*Δ cells was decreased even further. On the other hand, Tor1p level in *leu2leu4*Δ cells was slightly lower than that in *leu2* cells (Figure 3A). These decreased Tor1p levels in *LEU2*, *leu4*Δ, and *leu2leu4*Δ cells correspond well with the CLS extension compared to *leu2* cells (Figure 1A, 1C).

LEU2 and *leu4*Δ up-regulate cytochrome C oxidase subunit 2 (Cox2p) expression leading to a transient increase in ROS generation

In yeast, CLS extension by reduced Tor1p signaling was shown to be due to increased mitochondrial metabolism and expression of mitochondrial DNA-encoded genes[19]. The terminal component of the eukaryotic electron transport chain is cytochrome C oxidase. Among the twelve subunits that form the yeast cytochrome C oxidase, Cox1p, Cox2p, and Cox3p are the three largest subunits[25], and are encoded by mitochondrial DNA. Their enhanced translation is known to cause increased mitochondrial respiration, which in turn extends CLS. Cox2p and Cox3p levels are increased in *tor1*Δ strain[19], and Cox2p levels are regulated by nutritional availability and alanine metabolism[4]. To examine whether the effect of *LEU2* and *leu4*Δ on CLS extension is related to Cox2p levels, the amount of Cox2p in *LEU2*, *leu2*, *leu4*Δ, and *leu2leu4*Δ cells was analyzed. Cox2p levels were much higher in both *LEU2* and *leu4*Δ cells compared to that in *leu2* and *leu2leu4*Δ cells (Figure 3B) and were inversely correlated to the amount of Tor1p. On the other hand, Cox2p expression in *leu2leu4*Δ cells was significantly higher than that in *leu2* cells, which corresponds to the increased CLS of *leu2leu4*Δ cells compared to *leu2* cells.

Although production of toxic ROS has been suggested to be the main cause of aging, a growing number of

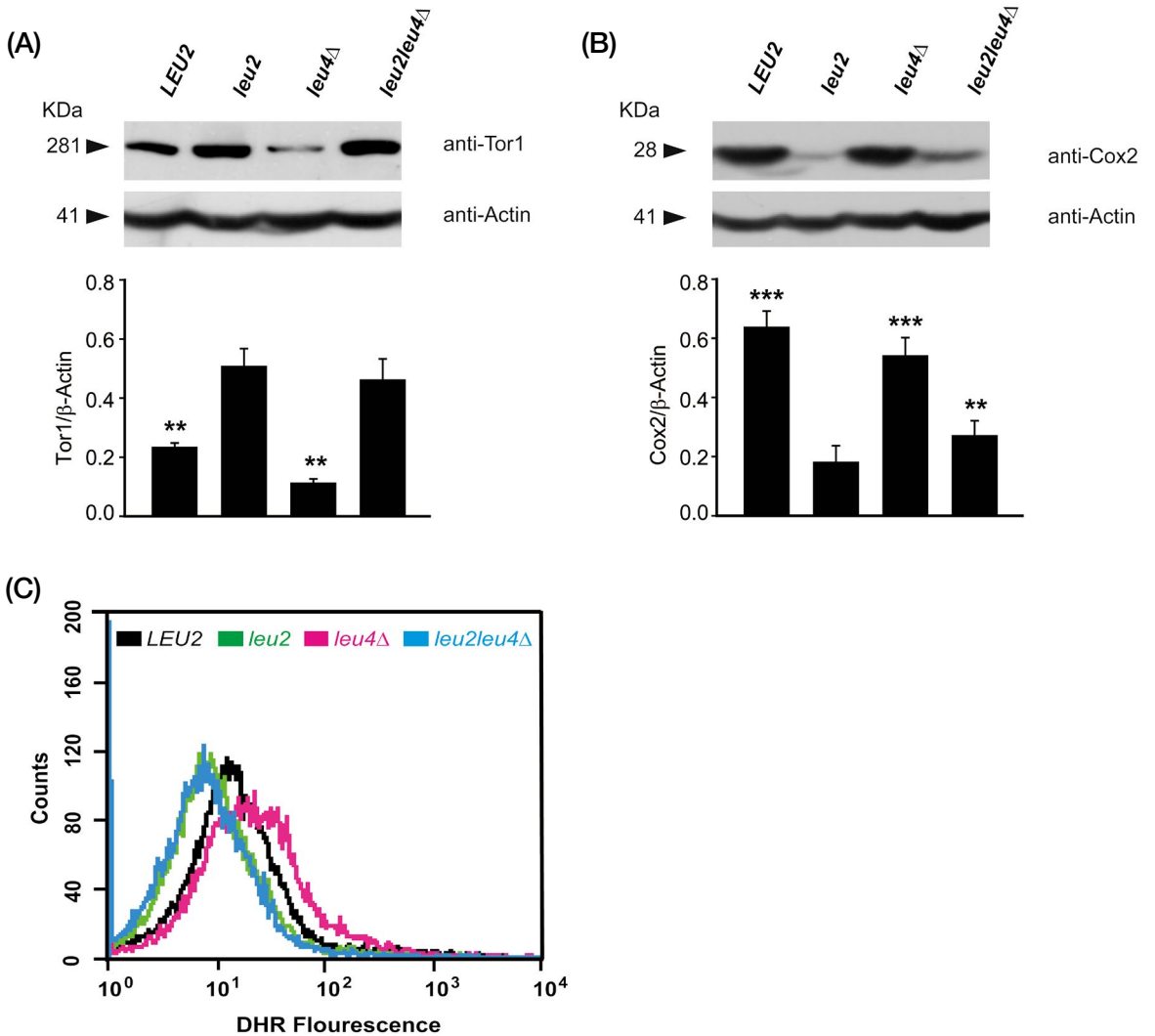

Figure 3. Effect of *leu4*Δ on Tor1p, Cox2p, and ROS levels. (A) Western blot analysis of Tor1p expression. *LEU2* cells express slightly but significantly decreased amount of Tor1p compared to *leu2* cells. On the other hand, Tor1p expression is significantly decreased in the absence of *LEU4*. However, Tor1p levels in *leu2leu4*Δ cells are similar to that in *leu2* cells. Bar graphs showing the levels of Tor1p normalized by the levels of actin. Data are shown as means of 3 independent experiments. $^{**}P \leq 0.01$ compared to *leu2*. (B) Western blot analysis of Cox2p expression. Cox2p expression in *LEU2* cells significantly increased compared to that in *leu2* cells. Cox2p expression in *leu4*Δ cells is similar to that in *LEU2* cells. However, the Cox2p level in *leu2leu4*Δ cells is much lower than that in *LEU2* cells. Bar graphs showing the levels of Cox2p normalized by the levels of actin. Data are shown as meansof 5 independent experiments. $^{**}P \leq 0.01$, $^{***}P \leq 0.001$ compared to *leu2*. (C) FACS analysis of cellular ROS levels. The ROS levels are slightly increased in *LEU2* and *leu4*Δ cells compared to *leu2* and *leu2leu4*Δ cells. In *leu4*Δ cells, the ROS level is further increased compared to that in *LEU2* cells. These results correspond well to decreased Tor1p expression and extended CLS of *LEU2* and *leu2leu4*Δ cells.

studies suggest that increased ROS in low doses also serves as a signal for various cellular mechanisms to promote health and longevity[26]. In addition, the increased lifespan in mice supplemented with BCAA was related to an up-regulated ROS defense system[5]. In yeast, reduced TOR signaling causes mitochondrial ROS signal generation that epigenetically changes the expression of nuclear genes[20]. Thus, decreased Tor1p expression and CLS extension in *LEU2* and *leu4* cells could also be related to changes in cellular ROS generation and Cox2p levels. FACS analysis revealed slightly increased ROS levels in *LEU2* and *leu4*Δ cells compared to that in *leu2* and *leu2leu4*Δ cells. Furthermore, ROS levels in *leu4*Δ cells were even higher than in *LEU2* cells (Figure 3C). The slightly increased ROS levels in *LEU2* and *leu4*Δ cells compared to that

in *leu2* cells might function as a signal to increase the ROS defense system.

Discussion

Leucine is known to link mitochondrial respiration, cell death, and CLS extension. Increased leucine availability supports proficient respiration and CLS extension[14,22,23]. Thus, our observation of a higher CLS in *LEU2* compared to that in *leu2* yeast could be the result of increased cellular leucine levels in *LEU2* cells. A recent study also suggested that cellular leucine levels would be higher in *leu4*Δ cells. During leucine biosynthesis, many genes in the pathway including *LEU2* and *LEU4*, are feedback regulated by the end product, leucine[15]. Both *LEU4* and *LEU9* encode a functional α-IPM synthase. However, Leu9p is resistant to leucine feedback inhibition while Leu4p is leucine sensitive[21]. Since Leu9p is fully functional as α-IPM synthase, Leu9p can continuously convert 2-KIV to α-IPM so that leucine might be continuously produced, regardless of the cellular leucine pool. Consequently, cellular leucine might accumulate to higher levels in *leu4*Δ cells than in *LEU2* cells. Thus, based on the study by Lopez *et al*.[21], the extended CLS of *leu4*Δ cells could be interpreted as the result of increased cellular leucine levels.

Nevertheless, how *leu4*Δ cells exhibit a further extended CLS than *LEU2* cells is difficult to understand because *leu4*Δ cells might produce less leucine than *LEU2* cells as most α-IPM synthase activity is provided by mitochondrially localized α-IPM which is encoded by *LEU4*. Indeed, the CLS of the *leu2leu4*Δ strain is much higher than that of *leu2* cells (Figure 1C). The extended CLS of *leu2leu4*Δ cells over *leu2* cells can not be the result of increased cellular leucine levels. Rather, this result suggests that another function of *LEU4* plays a role in longevity control in yeast. The common feature between *LEU2* and *leu4*Δ cells is a decreased accumulation of α-IPM. α-IPM does not accumulate in *LEU2* cells because of efficient α-IPM to KIC turnover. In *leu4*Δ cells, α-IPM would not accumulate because of decreased α-IPM synthesis due to the lack of the main α-IPM synthase, Leu4p. In the same vein, among the strains examined in this study, the α-IPM level might be lowest in *leu4*Δ cells, followed by *LEU2* cells. On the other hand, in *leu2* cells, leucine biosynthesis intermediates could accumulate due to the lack of β-IPM to KIC conversion. Therefore, the correlation between cellular α-IPM levels and CLS can explain the CLS pattern of *leu2leu4*Δ cells. In *leu2leu4*Δ cells, α-IPM levels would be higher than in *LEU2* cells because of the lack of β-IPM to KIC conversion by the *leu2* mutation, but would be lower than in *leu2* cells due to the lack of the major α-IPM synthase. The differential amount of α-IPM accumulation in the different mutant cells might be the factor that controls CLS via transcriptional regulation along with Leu3p.

On the other hand, the slightly increased ROS level in *LEU2* and *leu4*Δ cells is likely a hormetic phenomenon. Reduced Tor1p in those cells would slightly stimulate ROS generation which could stimulate Cox2p expression and prevent cellular damage. Previously, we reported that significantly increased ROS levels are the cause of increased cell death and severely decreased CLS in the alanine transaminase mutant, *alt1*Δ, constructed in the *leu2* background[4]. Although ROS levels in *LEU2* and *leu4*Δ cells were higher than that in *leu2* cells (Figure 3C), the levels were much lower than what was observed in *alt1*Δ cells. In fact, ROS levels in *LEU2* and *leu4*Δ cells are similar to that of *leu2* cells grown in CR conditions, indicating that leucine metabolism contributes to ROS changes, which in turn functions as a signal to increase the beneficial effects on cell survival. The effects of *LEU2* and *leu4*Δ are similar to hormesis whereby a low dose simulation results in beneficial effects. It is widely known that ROS at higher doses increases susceptibility to cell death, but low doses of ROS promote stress resistance and longevity. The lifespan extension by moderately increased ROS is thought to be due to the induction of healthy stimulations and a defense mechanism that would reduce harmful ROS generation and increase lifespan[26]. Since mitochondria are the main intracellular source of ROS, effective cellular respiration by increased expression of respiratory components could be another mechanism for lifespan extension in WT and *leu4*Δ yeast. One of these respiratory components is Cox2p which was increased in *LEU2* and *leu4*Δ cells. The slightly increased ROS in *LEU2* and *leu4*Δ cells may have caused the high expression of Cox2p, which eventually inhibited production of harmful ROS.

Conclusion

In summary, we observed that *LEU2* and *leu4*Δ cells exhibit CLS, Cox2p and Tor1p expression, and ROS level patterns similar to yeast grown in CR conditions[4]. These results suggest that *LEU2* and *leu4*Δ cells possess CR-like effects on CLS. Also, *LEU2* and *LEU4* gene products function in longevity control via TOR and cellular ROS signaling. On the other hand, the CLS of *leu2leu4*Δ cells indicates a rather complex role

of *LEU4* in CLS regulation. *leu2leu4*Δ cells cannot grow in the absence of exogenous leucine supplementation (Figure 1B), which suggests no leucine synthesis in the cell. If the cellular leucine level is a pivotal determinant of CLS, *leu2leu4*Δ would display a similar CLS to *leu2* cells. However, *leu2leu4*Δ cells exhibited a much higher CLS than *leu2* cells. These results suggest that extended CLS by *leu4*Δ is not simply due to increased cellular leucine levels and that *LEU2* positively contributes to yeast CLS, while *LEU4* negatively contributes to CLS, and their effects on CLS are independent of each other.

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Conflict of Interest Choco Michael Gorospe, Sung-Lim Yu, Mi-Sun Kang & Sung-Keun Lee declare that they have no conflict of interest.

Human and animal rights The article does not contain any studies with human and animal and this study was performed following institutional and national guidelines.

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