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GABA metabolism pathway genes, *UGA1* and *GAD1*, regulate replicative lifespan in *Saccharomyces cerevisiae*

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ABSTRACT

Many of the genes involved in aging have been identified in organisms ranging from yeast to human. Our previous study showed that deletion of the *UGA3* gene—which encodes a zinc-finger transcription factor necessary for γ-aminobutyric acid (GABA)-dependent induction of the *UGA1* (GABA aminotransferase), *UGA2* (succinate semialdehyde dehydrogenase), and *UGA4* (GABA permease) genes—extends replicative lifespan in the budding yeast *Saccharomyces cerevisiae*. Here, we found that deletion of *UGA1* lengthened the lifespan, as did deletion of *UGA3*; in contrast, strains with *UGA2* or *UGA4* deletions exhibited no lifespan extension. The Δ*uga1* strain cannot deaminate GABA to succinate semialdehyde. Deletion of *GAD1*, which encodes the glutamate decarboxylase that converts glutamate into GABA, also increased lifespan. Therefore, two genes in the GABA metabolism pathway, *UGA1* and *GAD1*, were identified as aging genes. Unexpectedly, intracellular GABA levels in mutant cells (except for Δ*uga2* cells) did not differ from those in wild-type cells. Addition of GABA to culture media, which induces transcription of the *UGA* structural genes, had no effect on replicative lifespan of wild-type cells. Multivariate analysis of ^{1}H nuclear magnetic resonance spectra for the whole-cell metabolite levels demonstrated a separation between long-lived and normal-lived strains. Gas chromatography–mass spectrometry analysis of identified metabolites showed that levels of tricarboxylic acid cycle intermediates positively correlated with lifespan extension. These results strongly suggest reduced activity of the GABA-metabolizing enzymes extends lifespan by shifting carbon metabolism toward respiration, as calorie restriction does.

1. Introduction

Many genes involved in aging have been identified in a variety of organisms, including mice, nematodes, and yeasts [1–3]. Nutrient and stress sensors modulate lifespan in response to many different environmental and physiological signals. Reduced activity of nutrient-sensing pathways or dietary restriction slows aging and increases lifespan [4]. The nutrient-sensing pathways include the kinase target of rapamycin (TOR), AMP-activated protein kinase (AMPK), sirtuins, and insulin/insulin-like growth factor (IGF-1) signaling, among others [5], and these nutrient signaling molecules also regulate stress response pathways [6].

In the budding yeast *Saccharomyces cerevisiae*, two types of cellular aging have been proposed: replicative and chronological. Replicative lifespan is defined as the number of daughters produced by each dividing mother cell and may be similar to aging of mitotically active cells in multicellular organisms [7]. Chronological lifespan is the length of time a population remains viable in the post-diauxic and stationary phases [8]. Replicative lifespan is regulated by genes encoding metabolic enzyme, including *HXK2*, encoding hexokinase isoenzyme 2; *ADH1*, encoding alcohol dehydrogenase; and *LYS9*, encoding saccharopine dehydrogenase, in addition to the nutrient and stress sensing genes [3,9,10]. The effects of metabolic enzyme genes on lifespan regulation in higher eukaryotes are not known, thus yeast is a unique and powerful model for investigation of the effects of these genes on lifespan in eukaryotic cells.

In our previous study, fingerprinting of identified compounds using gas chromatography–mass spectrometry (GC–MS) and capillary electrophoresis-mass spectrometry revealed a correlation between replicative lifespan and metabolic profile in *S. cerevisiae* [11]. The levels of amino acids (e.g., glutamine and proline) and nucleotide derivatives (e.g., inosine and cAMP) correlated most closely with replicative lifespan. Furthermore, we established a multivariate model to predict lifespan from a metabolic profile

Abbreviations: GABA, γ-aminobutyric acid; TCA, tricarboxylic acid; GC–MS, gas chromatography–mass spectrometry; NMR, nuclear magnetic resonance; OPLS-DA, orthogonal projection on latent structure-discriminant analysis.

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and demonstrated that three genes—utilization of γ-aminobutyric acid (GABA) (*UGA)3*, *FZF1*, and *URH1*—are aging-related genes. Disruption of *UGA3*, *FZF1*, or *URH1* resulted in lifespan extension.

The *UGA3* gene encodes a zinc-finger transcription factor necessary for GABA-dependent induction of the *UGA* structural genes, *UGA1*, *UGA2*, and *UGA4* [12,13]. Uga1p (GABA aminotransferase) deaminates GABA to succinate semialdehyde. Uga2p (succinate semialdehyde dehydrogenase) converts succinate semialdehyde to succinate, which is supplied to tricarboxylic acid (TCA) cycle. Uga4p (GABA permease) catalyzes the transport of GABA to the vacuole. These *UGA* structural genes are involved in the use of GABA as a nitrogen source [12]. Uga3p is required for transcriptional induction of the *UGA* structural genes in the presence of GABA and maintenance of basal transcription of the *UGA* genes in the absence of GABA [14].

To understand the cause of the replicative lifespan increase in the *UGA3* deletion mutant, we tested deletions of each *UGA* structural gene that is under the control of Uga3p for effects on replicative lifespan. Deletion of *UGA1*extended lifespan, but deletion of *UGA2* or *UGA4* did not. We also found that *GAD1*, which encodes a GABA-metabolizing enzyme, was also involved in aging. Based on our findings, we discuss the hypothesis that reduced activity of the GABA-metabolizing enzymes increases lifespan by shifting carbon metabolism toward respiration.

2. Materials and methods

2.1. Strains and media

All *S. cerevisiae* strains used in this study were derived from BY4742 (*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*). Deletion strains were obtained from the MATα ORF deletion collection (Open Biosystems, USA). Yeast extract (1%), bactopeptone (2%), dextrose (2%) (YPD) medium was used for routine cultures.

2.2. Lifespan determination

Replicative lifespan was assayed with minor modifications as described previously [15]. Yeast cells were thawed from frozen stock and streaked onto YPD agar plates or YPD plates containing 200 μg/ml G418. After 3 days, a single colony was transferred to 2 ml of YPD liquid media, and cells were grown overnight. The culture was diluted 1:500 in fresh YPD liquid medium, and a sample was spread onto YPD agar plate containing 10 μg/ml phloxine B. YPD agar plates containing GABA (0.1% or 0.5%) were used as indicated in the text. Using a micromanipulator, 48 cells were arrayed on a YPD plate and allowed to undergo one or two divisions. Virgin cells were then selected and subjected to lifespan analysis. Except during manipulation, plates were sealed with Parafilm and incubated at 30 °C during the day and stored at 4 °C at night to avoid excessive budding. Daughter cells were removed by gentle agitation with a dissecting needle and scored every 2 h. For each of the 48 cell lines, buds from each mother cell were counted until division of living cells ceased or cells were stained with phloxine B. This assay was performed at least twice with each strain. For statistical analysis, lifespan data sets were compared by a Wilcoxon rank-sum test. Two strains are stated to have a significant difference in lifespan when $P < 0.01$.

2.3. GC–MS analysis

Sample preparation from dried yeast cells and GC–MS analysis were performed as described previously [11]. GC–MS analysis was performed independently five times for each strain. For comparison of intracellular levels of GABA, succinic acid, and glutamic acid, the area under the peak representing the compound was measured. To process GC data, raw chromatographic data (Pegasus file, ∗.peg) were converted into ANDI files (Analytical Data Interchange Protocol, ∗.cdf). The ANDI formatted data was converted and transferred between different mass spectral instruments. Baseline correction, peak detection, and peak alignment was performed with the free software MetAlign (Wageningen UR, The Netherlands, freely available at http://www.pri.wur.nl/UK/products/MetAlign/). The distinctive *m*/*z* peak for each compound was normalized on the basis of the intensity of the ribitol peak. Each metabolite was identified on the basis of an in-house chemical library. An *m*/*z* peak was selected as a distinctive *m*/*z* peak in the mass spectrogram if its intensity was not affected by neighboring peaks.

2.4. ^{1}H-NMR analysis

Extraction of metabolites from yeast cells was performed as described previously [16]. Yeast cell cultures (25 ml) were inoculated at 4.0×10^6 cells/ml and grown between 5 and 6 h to reach an OD of 1.0. The cells were isolated from culture medium by centrifugation (5 min; 1750×*g*), washed in water once. Then, 2 ml of 75% (v/v) ethanol solution, at 80 °C, was added directly to the cell pellet. This mixture was mixed by vortex for 30 s and heated for 3 min to 80 °C. The mixture was cooled on ice for 3 min and subsequently dried in rotary vacuum concentrator at 35 °C.

^{1}H-NMR analysis was performed as described previously [17]. The dried extracts were dissolved in 0.6 ml of 0.1 M potassium phosphate buffer, pH 7.0, in D_2O, containing 1 mM sodium 3-trimethylsilyl-2,2,3,3-tetradeuteropropionate (TSP), mixed by vortex, and then centrifuged at 16,000*g* for 10 min to remove material that had not dissolved. ^{1}H-NMR spectra were acquired at 400 MHz into 4096 data points using a pulse angle of 90°, an acquisition time of 0.8 s, and a sweep width of 5 kHz. The overall pulse repetition time was 5 s. The samples were spun at 16 Hz and maintained at 30 °C during data acquisition. The spectra were the sum of 128 transients. The chemical shift scale was referenced to the signal from TSP at 0.0 ppm.

Reduction of date from ^{1}H-NMR analysis was carried out by binning the spectra into 0.05 ppm regions; i.e., each spectrum was divided into 0.05 ppm regions, and the total signal within each region was integrated between 0.0 and 10 ppm. The region from 4.4 to 5.5 ppm, which contained the residual signal from the water resonance, was excluded. This resulted in each spectrum being reduced to a vector of length 176.

2.5. Multivariate analysis

The data sets from ^{1}H-NMR and GC–MS analyses were judged in all cases by orthogonal projection on latent structure-discriminant analysis (OPLS-DA) using SIMCA-P+ 12.0.1 (Umetrics, Sweden).

3. Results and discussion

3.1. The UGA1 gene encoding GABA aminotransferase regulates replicative lifespan

Deletion of the *UGA3* gene extends replicative lifespan; *UGA3* deletion mutant budding yeast cells produce about 60% more buds than wild-type cells [11]. The *UGA3* gene encodes a zinc-finger transcription factor that is required for induction of *UGA1*, *UGA2*, and *UGA4* when GABA is present in culture medium [12,14]. Even in the absence of GABA, deletion of *UGA3* down-regulates the basal transcription of *UGA1* [14]. We therefore expected that down-regulation of the Uga3p target genes would cause increased lifespan.

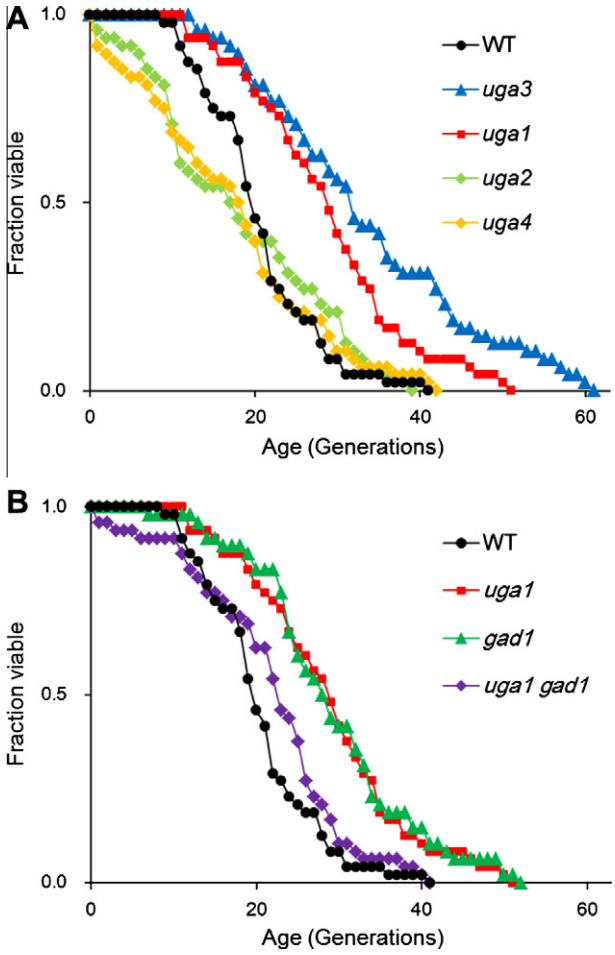

Fig. 1. Replicative lifespans of cells carrying a deletion in *UGA1* or *GAD1*, genes encoding GABA-metabolizing enzymes. (A) Lifespan of deletion mutants of Uga3p target genes was determined. (B) Lifespan of the *GAD1* deletion mutant and the *UGA1 GAD1* double mutant was determined. Replicative lifespan was measured at least twice for each strain, and one measurement for each strain is shown. Average lifespans: BY4742 (wild type, WT), 20.7 generations; Δ*uga3*, 33.7; Δ*uga1*, 28.9; Δ*uga2*, 18.4; Δ*uga4*, 17.4; Δ*gad1*, 29.3; Δ*uga1* Δ*gad1*, 21.9.

To determine whether *UGA1*, *UGA2*, and/or *UGA4* were involved in aging, we measured the replicative lifespan of each individual deletion mutant (Fig. 1A). Deletion of *UGA1* resulted in an increase in an average replicative lifespan of approximately 40% compared with the wild-type strain BY4742, while no increase in lifespan was observed for Δ*uga2* or Δ*uga4* mutants. Therefore, we concluded that the *UGA1* gene, which encodes the GABA aminotransferase that deaminates GABA to succinate semialdehyde, negatively regulated replicative lifespan. These findings suggested that longevity of the Δ*uga3* mutant was caused by transcriptional down-regulation of *UGA1*, but not by changes in *UGA2* and *UGA4* regulation.

This study is the first to demonstrate that a GABA aminotransferase gene regulated aging, although there are age-related changes in GABA transaminase (GABA-T) immunoreactivity in the hippocampus and dentate gyrus of the gerbil [18]. GABA-T protein content is elevated in gerbils in postnatal month 12, but levels decreased after that point. It is unclear whether expression of GABA aminotransferase changes as yeast cells age.

3.2. The GAD1 gene encoding glutamate decarboxylase regulates replicative lifespan

Because Uga1p catalyzes deamination of GABA, we investigated another GABA-metabolizing enzyme gene (*GAD1*) that encodes glutamate decarboxylase, which converts glutamate into GABA [19]. Deletion of *GAD1* resulted in an increase in average lifespan of approximately 40%, similar to that of *UGA3* and *UGA1* (Fig. 1B). We concluded that the *GAD1* gene also negatively regulated replicative lifespan. These results indicated that the GABA-metabolizing enzyme genes, *UGA1* and *GAD1*, are aging genes.

In human visual cortex, expression of the GABA synthesizing enzyme (GAD65), which is the homolog of yeast Gad1p, increases from early in life to the teen and adult years, and GAD65 levels decline in older adults [20]. Although the studies on aging-related expression of yeast *GAD1* gene have not been published, transcription of the *GAD1* gene is regulated by Mtl1p, which activates general stress response during glucose starvation and oxidative stress via the transcription factor Msn2p/Msn4p [21]. Therefore, glutamate decarboxylase Gad1p might regulate lifespan in response to nutrient limitation and/or oxidative stress.

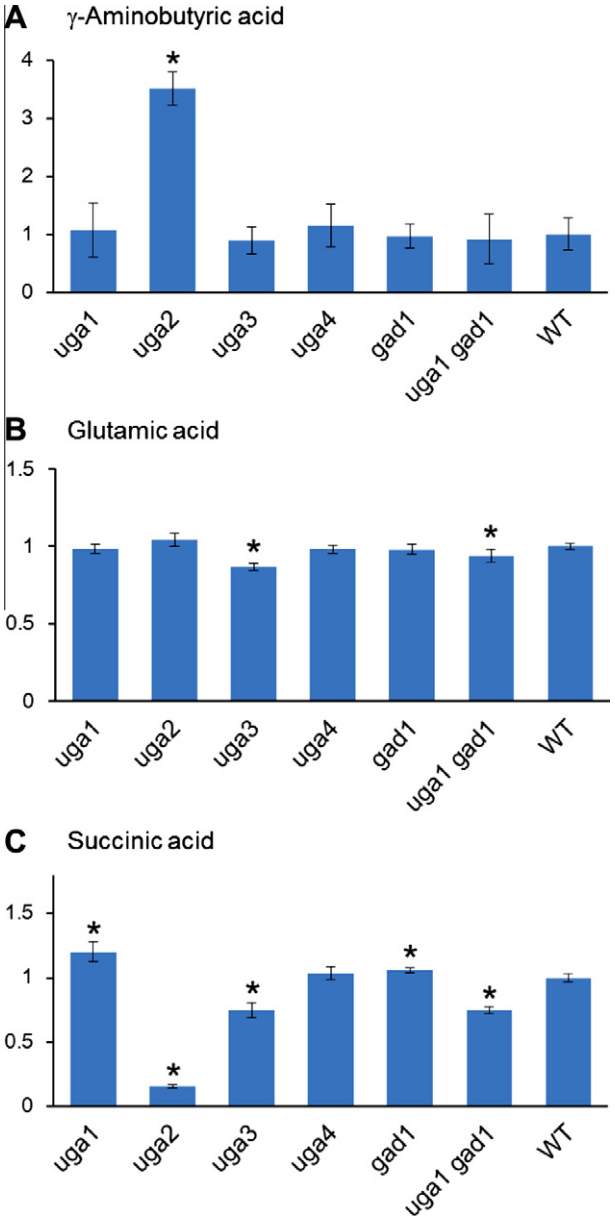

Fig. 2. Comparison of intracellular levels of GABA metabolites in mutants carrying deletions of genes in the GABA metabolic pathway. (A) GABA. (B) glutamic acid. (C) succinic acid. Data are expressed as mean ± SEM. $^{*}P < 0.05$ ($n = 5$).

The increase in lifespan associated with the *UGA1* and *GAD1* deletion mutants led us to speculate that these deletions may additively increase lifespan. We constructed a Δ*uga1* Δ*gad1* double deletion mutant strain by tetrad dissection and determined its replicative lifespan. Contrary to our expectation, lifespan of the double mutant was shorter than that of the Δ*uga1* and Δ*gad1* single mutants and comparable to that of the wild-type strain (Fig. 1B).

3.3. Supplementation of GABA to culture media has no effect on lifespan

Addition of 0.1% GABA to culture media induces transcription of the *UGA1*, *UGA2*, and *UGA4* genes [12,14], and we measured replicative lifespan of the wild-type strain on YPD plate medium supplemented with GABA. There was no significant difference between lifespan on GABA-containing and GABA-free media for wild-type cells. The mean lifespan on 0.1% GABA medium was 21.7 ± 8.9 generations and the maximal lifespan was 44 generations while the mean lifespan on YPD medium without GABA 22.4 ± 8.4 and the maximal lifespan 42. When GABA concentration was increased to 0.5%, the mean lifespan was 21.4 ± 7.2 generations and the maximal lifespan was 41 generations. Therefore, we concluded that addition of GABA sufficient to activate transcription of the *UGA* structural genes had no effect on lifespan. Interestingly, increased transcription of the Uga3p target genes seemed not to affect lifespan, while deletion of *UGA3* did. This result was similar to the case of a *RAS1* deletion; *RAS1* encodes a GTPase involved in G-protein signaling in the adenylate cyclase activating pathway. A *RAS1* deletion increases lifespan, but overexpression of *RAS1* does not result in a change in lifespan [22]. To our knowledge, there is no report indicating that GABA supplements extend lifespan in any organism, including yeast.

3.4. The metabolomic profile of GABA-metabolizing enzyme mutants correlates with replicative lifespan

To investigate the reason for the increase in lifespan of Δ*uga1* and Δ*gad1* cells, we measured the metabolites in the GABA metabolic pathway (GABA, glutamic acid, and succinic acid) in the long-lived (Δ*uga3*, Δ*uga1* and Δ*gad1*) and normal-lived (Δ*uga2*, Δ*uga4*, Δ*uga1* Δ*gad1*, and wild type) strains using GC–MS analysis. The intracellular GABA levels of all deletion mutants except for Δ*uga2* were comparable to that of the wild-type strain (Fig. 2A). This result indicated that intracellular GABA levels did not regulate replicative lifespan. There was almost no difference in intracellular level of glutamic acid, the substrate of Gad1p, between strains; however, there was a slight decrease in Δ*uga3* and Δ*uga1* Δ*gad1* cells (Fig. 2B). This observation was consistent with the observation from our previous study that glutamic acid levels do not correlate with lifespan extension [11]. Succinic acid levels were significantly lower in the *UGA2* deletion mutant. Interestingly, the level of succinic acid in the long-lived Δ*uga1* and Δ*gad1* mutants was elevated (Fig. 2C), suggesting that succinic acid might be a key contributor to lifespan extension. Low levels of succinic acid in the long-lived Δ*uga3* mutant may be a consequence of down-regulating *UGA2* expression.

Analysis of levels of the metabolites in the GABA metabolic pathway did not reveal a striking correlation between longevity and metabolite levels in the *UGA* mutants; therefore, the whole-cell metabolite levels were surveyed by ^{1}H-NMR-based metabolomic

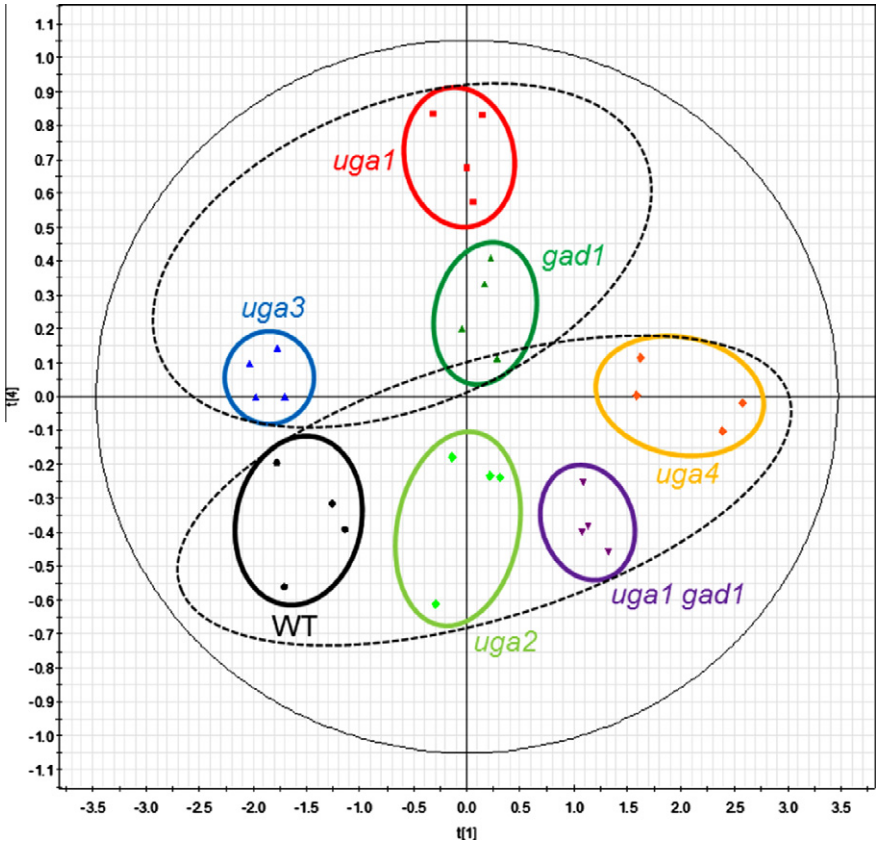

Fig. 3. Orthogonal projection on latent structure-discriminant analysis (OPLS-DA) of ^{1}H-NMR spectra of GABA metabolic pathway mutants. OPLS-DA score plot (axis 1 vs. axis 4) is indicated. Plots from each strain coalesced into a cluster as shown by a circle. OPLS axis four separated the clusters into two groups, the long-lived mutants (Δ*uga3*, Δ*uga1* and Δ*gad1*) and the strains with ordinary lifespans (wild type, Δ*uga2*, Δ*uga4* and Δ*uga1* Δ*gad1*), and each group is encircled by a dashed line.

analysis. We prepared cell extracts from mid-logarithmic phase yeast liquid cultures of the long-lived mutants (Δ*uga3*, Δ*uga1* and Δ*gad1*), mutants with normal lifespans (Δ*uga2*, Δ*uga4* and Δ*uga1* Δ*gad1*), and the wild-type strain grown in YPD and measured ^{1}H-NMR spectra independently four times (Supplementary data 1). The NMR-derived metabolomic profile was visualized by orthogonal projection on latent structure-discriminant analysis (OPLS-DA) (Fig. 3). OPLS-DA score plot indicated that all four plots from each mutant strain coalesced into clusters. We found that the clusters separated into two large groups and that group position along axis 4 correlated with lifespan. One group contained the three long-lived mutants, Δ*uga3*, Δ*uga1*, and Δ*gad1*. The other contained the strains with normal lifespans, Δ*uga2*, Δ*uga4*, Δ*uga1* Δ*gad1*, and wild type. These results indicate that the GABA metabolic pathway mutants have metabolomic profiles that correlate with replicative lifespan.

3.5. Tricarboxylic acid cycle intermediates contribute to lifespan extension

Our data indicated that succinic acid contributed to lifespan extension and that the metabolomic profile from ^{1}H-NMR spectra correlated with longevity. To identify other specific metabolites that may affect lifespan, we identified and measured 57 compounds by GC–MS analysis (Supplementary data 2) and examined correlations between levels of individual metabolites and replicative lifespan. The GABA metabolic pathway mutants were classified into the long-lived and normal-lived mutants and OPLS-DA was performed using the GC–MS data (Fig. 4). The scatter plot (S-plot) indicates levels of three neighboring tricarboxylic acid (TCA) cycle intermediates (succinic acid, fumaric acid, and malic acid) positively correlated with lifespan extension; however, citric acid and α-ketoglutaric acid, other TCA cycle intermediates, did not. Interestingly, 5-aminolevulinic acid, the first compound in the porphyrin synthesis pathway that leads to heme production, also positively correlated with lifespan extension.

Reportedly, calorie restriction extends replicative lifespan in budding yeast by increasing respiration [23]. In yeast, when glucose levels are high, fermentation takes precedence over respiration. When glucose is limiting, respiration takes precedence and carbon is shunted to the mitochondrial TCA cycle, thereby increasing electron transport and respiration. The *UGA1* and *GAD1* deletions may have caused a shift in carbon metabolism toward the TCA cycle and increased respiration, as calorie restriction does. A shift in carbon metabolism may cause the lifespan extension observed in Δ*uga1* and Δ*gad1* single mutants. Moreover, this hypothesis is consistent with the observation that the TCA cycle intermediates and 5-aminolevulinic acid did not increase in the Δ*uga1* Δ*gad1* double mutant, which displayed a normal lifespan. Our finding that mutations in two GABA-metabolizing enzyme genes (*UGA1* and *GAD1*) increased the level of TCA cycle intermediates was unexpected. A search for mutations in genes encoding metabolic enzymes that increase respiration may lead to the identification of additional aging genes.

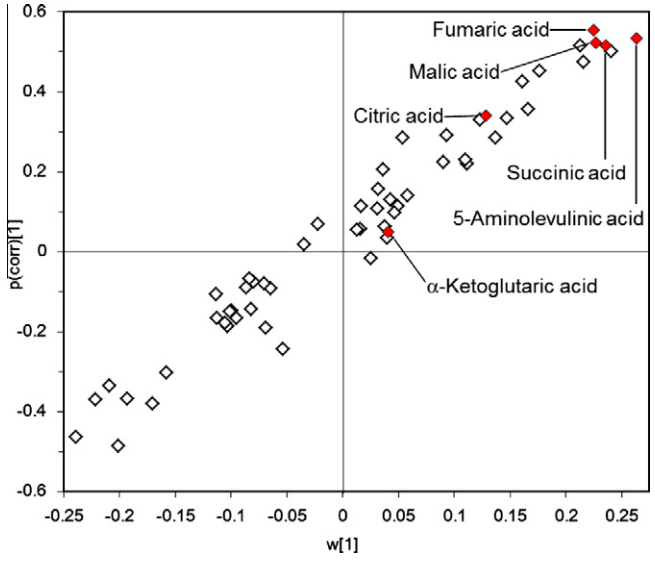

Fig. 4. Scatter plot (S-plot; loading (*w* [1]) vs. correlation (p(corr)[1])) of OPLS-DA of GC–MS metabolite profiles from GABA metabolic pathway mutants. Plot positions of TCA cycle intermediates (succinic acid, malic acid, fumaric acid, citric acid, and α-ketoglutaric acid) and 5-aminolevulinic acid are indicated.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2011.02.136.

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