Tor-Sch9 deficiency activates catabolism of the ketone body-like acetic acid to promote trehalose accumulation and longevity

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Summary

In mammals, extended periods of fasting leads to the accumulation of blood ketone bodies including acetocacetate. Here we show that similar to the conversion of leucine to acetacetate in fasting mammals, starvation conditions induced ketone body-like acetic acid generation from leucine in S. cerevisiae. Whereas wild-type and ras2Δ cells accumulated acetic acid, long-lived tor1Δ and sch9Δ mutants rapidly depleted it through a mitochondrial acetate CoA transferase-dependent mechanism, which was essential for lifespan extension. The sch9Δ-dependent utilization of acetic acid also required coenzyme Q biosynthetic genes and promoted the accumulation of intracellular trehalose. These results indicate that Tor-Sch9 deficiency extends longevity by switching cells to an alternative metabolic mode, in which acetic acid can be utilized for the storage of stress resistance carbon sources. These effects are reminiscent of those described for ketone bodies in fasting mammals and raise the possibility that the lifespan extension caused by Tor-S6K inhibition may also involve analogous metabolic changes in higher eukaryotes.

Key words: acetic acid; aging; chronological lifespan; leucine; Sch9.

Introduction

The mechanisms underlying the aging process in mammals remain poorly understood. The budding yeast Saccharomyces cerevisiae has served as a primary model organism to study the mechanisms of aging. For instance, yeast has been responsible for the identification of two of the major pro-aging pathways: the Tor/S6K and the Ras/cAMP/PKA pathways (Pedruzzi et al., 2003; Wei et al., 2008). In fact, yeast cells are nonviable if they lack both Ras1 and Ras2 or Tor1 and Tor2 (Toda et al., 1985; Barbet et al., 1996).

Because high level of acetic acid promotes apoptosis in yeast, others have proposed that acetic acid and not ethanol is the primary factor promoting culture acidification, chronological aging, and apoptotic death (Burtner et al., 2009). It has also been proposed that CR increases chronological survival by reducing extracellular acetic acid and that the longevity of sch9Δ and ras2Δ mutants is due to reduced acetic acid production during the proliferative phase (sch9Δ) and acetic acid resistance after nutrient exhaustion (sch9Δ and ras2Δ) (Burtner et al., 2009). We instead provide strong evidence that, at physiological levels, acetic acid promotes aging simply by activating pro-aging pathways in yeast, analogously to glucose and ethanol.

In this study, we investigated the role of acetic acid in chronological aging and examined its source and impact on the lifespan of wild-type and long-lived S. cerevisiae.

Results

Yeast mutants deficient in the Ras/cAMP/PKA and Tor/Sch9 pathways exhibit different patterns of ethanol and acetic acid accumulation during chronological aging

To establish the relationship between carbon source metabolites and CLS, we measured the levels of ethanol and acetic acid in chronologically aged yeast cultures (Fig. 1). Wild-type cells accumulated acetate during chronological aging, but the depletion of either TOR1 or SCH9 resulted in depletion of extracellular acetate after a small peak on day 3 (Fig. 1A). These data indicate that the Tor/Sch9 pathway blocks the utilization of acetate. By contrast, mutations in RAS2 or CYR1, the central components of the other yeast pro-aging pathway with orthologs that promote aging in mice (Ferbeyre et al., 2002; Yan et al., 2007), accumulated similar or even higher levels of acetate compared to the wild-type strain (Fig. 1A). These results suggest that during the early stages of lifespan, as extracellular glucose and other nutrients become depleted, the absence of Tor/Sch9 signaling promotes a metabolic change that utilizes acetic acid, analogous to the generation and use of the ketone bodies, acetocetic acid and...
Tor-Sch9 deficiency activates ketone body-like catabolism in promoting longevity, J. Hu et al.

Concentration of ethanol (0.8%) or of acetic acid (50 mM) using the background (Tor-Sch9 deficiency activates ketone body-like catabolism in promoting longevity, J. Hu et al., 2012) did not shorten lifespan to the same level as 2% glucose or 100 mM acetic acid (Wei et al., 2009). To examine whether the carbon source influences stress resistance, we challenged cells with heat shock and oxidative stress (H2O2 treatment for 30 min) after switching them to various carbon sources for 24 h. At physiological concentrations, all the carbon sources sensitized yeast cells only to oxidative stress (Fig. 2B).

Gis1 and Msn2/4 are stress-response transcription factors required for lifespan extension of Tor/Sch9- or Ras/cAMP/PKA-deficient cells. To further understand how carbon sources affect the stress response, we employed STRE- and PDS-driven LacZ reporter gene assays to monitor Msn2/4 and Gis1 transactivation, respectively (Fig. 2C,D). High concentration of acetic acid (100 mM) completely repressed the STRE- and PDS-dependent transactivation, while its physiological concentrations, up to 50 mM, reduced the activation of these transcription factors (Fig. 2C,D). This effect could explain the cytotoxicity of super-physiological levels of acetic acid. Cells in glucose medium showed a significant reduction in PDS-driven gene expression in agreement with the stress resistance results (Fig. 2B,D). Based on these results, we conclude that physiological concentration of either acetic acid or ethanol in combination with media acidification similarly affects cellular protection and survival.

To determine the relative contribution of acidification to cellular survival and stress sensitization, we monitored the effect of pH on the activity of the Msn2/4 and Gis1 stress resistance transcription factors (Fig. 2E,F). Similar to the experiment above, we switched cells to MES buffer (pH 6.0 or 4.0) supplemented with either physiological level of ethanol (0.8%) or acetate (50 mM). Mild acidification (pH 4.0) did not significantly alter PDS- or STRE-dependent transactivation. In the presence of acetic acid, the increase in pH from 4 to 6 elevated STRE- and PDS-dependent LacZ activity (Fig. 2E,F). This observation may explain why buffering the acidic media to pH 6 extends longevity (Fabrizio et al., 2005; Burtner et al., 2009; Kaerberlein, 2010). By contrast, a pH increase promoted PDS- but not STRE-dependent LacZ activity in ethanol medium (Fig. 2E,F). We also buffered the spent medium collected from day 3 wild-type cultures to pH 6.0 and found that PDS-LacZ, but not STRE-LacZ, activity was elevated and reached an almost two-fold increase compared to cells in the unbuffered medium by 48 h (Figure S1A,B Supporting Information). These results suggest that Gis1 transactivation via the PDS elements, which are mostly negatively regulated by the Tor/Sch9 signaling pathway (Wei et al., 2008), may be responsible for part of the effects of medium acidification and ethanol or acetic acid on stress resistance and CLS.

In order to reconcile these data and the proposal by others that acetic acid is a toxic molecule that causes acute cytotoxicity (Kaerberlein, 2010; Longo et al., 2012), we investigated whether differences in technical procedures may be responsible for the different results. Using loose-fitting plastic caps that allow high aeration instead of the aluminum foil caps that limit aeration used in our method, Burtner et al. (2009) showed that ethanol was depleted rapidly and acetic acid accumulates in aging yeast cultures. We performed our viability assay using both methods. The evaporation rate was much higher in plastic cap-covered flasks as the volume decreased by 50–70% by day 11 compared to a 15% decrease in flasks with aluminum foil caps (data not shown). The higher aeration also caused early depletion of ethanol and caused the levels of acetic acid to reach a concentration close to 100 mM (Fig. 2G, H). The pH of wild-type cultures was maintained between 3.5 and 3.8 throughout the lifespan using both methods (Figure S1C Supporting Information). These results indicate that the methodology used by Burtner et al. caused early depletion of ethanol and increased the levels of acetic acid to a toxic range, in contrast to our method in which ethanol remains the major carbon source and the concentration of acetic acid reached between days 3 and 7 is approximately 50–60 mM. This

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demonstrates that CLS, similar to aging assays in other organisms, is dependent on environmental conditions.

Mutations that reduce acetic acid levels do not affect longevity or acidification

To further investigate the role of acetic acid in S. cerevisiae chronological aging, we compared the survival of isogenic yeast strains carrying mutations in genes involved in acetic acid metabolism (Fig. 3A). ADY2 encodes an acetate transporter, and its disruption has been reported to affect acetic acid cellular uptake (Paiva et al., 2004). PDC6 encodes an isoform of the pyruvate decarboxylase, which decarboxylates pyruvate to acetaldehyde. Deletion of PDC6 is expected to affect the production of acetaldehyde from pyruvate and, in turn, reduce acetate production (Pronk et al., 1996). ACS1 is an acetyl-coA synthetase, whose deficiency can affect acetate production (De Virgilio et al., 2004).

Fig. 2 Effect of carbon sources on chronological aging and stress response. (A) Chronological survival of wild-type (DBY746) cells in different media. Day 3 cells were washed and transferred to MES buffer in the presence of different carbon sources at the indicated concentrations. All media were adjusted to the same pH as the control (buffer only). (B) Stress resistance assay of wild-type strain (DBY746) 24 h after the switch to various buffers. Cells were heated for 100 min at 55 °C or treated with 70 mM H2O2 for 30 min. A representative experiment was shown. (C, D) LacZ reporter assay. Wild-type cells with chromosomally integrated STRE- or PDS-LacZ reporter genes were switched to buffer containing different carbon sources after three days of growth in standard SD medium. The STRE-LacZ (C) and PDS-LacZ (D) activities were measured 8 h and 24 h after the switch. Data shown are mean ± SEM (n = 3–4). (E, F) Wild-type (DBY746) cells were transferred to 40 mM MES buffer (pH 6, pH 4) supplemented with ethanol (0.8%) or acetic acid (50 mM) after two days of growth in SD medium. The STRE-LacZ (E) and PDS-LacZ (F) activities were measured 4, 8, and 24 h after the switch (n = 3). Star denotes P < 0.05; double stars denote P < 0.01. (G, H) Wild-type cells were grown in SD medium under the standard condition with limited aeration (aluminum caps) or high aeration condition (loose plastic caps). Extracellular acetate and ethanol concentrations were measured every other day.

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Acetic acid is a ketone body-like metabolite generated in part from leucine metabolism

In mammalian cells, ketone bodies, including acetone, acetoacetic acid, and β-hydroxybutyric acid, are generated from fatty acids and ketogenic amino acids catabolism. We hypothesized that acetic acid is a ketone body-like metabolite that serves as an energy source during periods of glucose depletion. We tested whether leucine could affect acetic acid levels. The commonly used laboratory yeast strains contain mutations in amino acid biosynthetic genes (see experimental procedures). Standard growth medium is supplemented with excess 4X amino acids (leucine, histidine, and uracil, in both backgrounds and tryptophan only for DBY746) to compensate for these genetic deficiencies (Fabrizio et al., 2001). When we reduced the excess amino acids or only leucine to 1X, the extracellular acetate concentration was markedly reduced, while ethanol levels were not affected (Fig. 4A,C). Notably, cell density of the 1X leucine culture reached only 25–30% of that reached by growth in 4X leucine (standard medium), but the acetic acid production remained greatly reduced when the effects were normalized per cell number (Figure S2A,B Supporting Information). Moreover, we evaluated CLS of DBY746 wild-type cells in 1X leucine media and observed that lifespan was reduced when compared to same cells grown in standard media (Figure S4A Supporting Information). Similar results were obtained with the BY4741 genetic background (Fig. 4B,D). Furthermore, the major difference in acetic acid production did not cause significant differences in culture pH, indicating that acetic acid is not a major factor in medium acidification (Fig. 4E,F). An increase in leucine levels could promote acetate production in the BY4741 (Figure S2C Supporting Information), but not in the DBY746 wild-type strain, whose extracellular acetate levels were already high (data not shown).

Bat1 and Bat2 are the mitochondria and cytosolic branched-chain amino acid (BCAA) aminotransferases (Fig. 4G) in the leucine metabolism pathway (Dickinson, 2000). Deletion of either of these two genes had little effect on acetate accumulation, but the double knockout mutants showed reduced extracellular acetate levels during early chronological aging (Fig. 4H). However, chronological survival of the bat1Δ bat2Δ double mutants in the standard SD medium did not differ significantly from those of wild-type cells in same media (Figure S4B Supporting Information). This observation is possible due to utilization of an alternative pathway in the bat1Δ bat2Δ double mutants. These data suggest that acetate production shares similarities to that of ketone bodies generation by leucine in mammals.

To examine whether prototrophy and auxotrophy may affect the survival of the long-lived mutants, we introduced the LEU2 gene in the tor1Δ and sch9Δ strains utilized in this study. As shown in Supplemental Figure S2D, both tor1Δ and sch9Δ mutants exhibit a longer lifespan in comparison with wild-type, with sch9Δ presenting a more pronounced extended CLS than tor1Δ.
The acetate utilization by the \textit{sch9}\textsuperscript{Δ} mutant is dependent on Ach1

The pro-longevity effect of Tor/S6K inhibition, demonstrated in yeast, worms, flies, and mice, is one of the most promising pharmacological interventions with the potential to extend healthy lifespan in humans (Fabrizio et al., 2001; Hansen et al., 2007; Harrison et al., 2009; Pan & Shadel, 2009; Katewa & Kapahi, 2011). To investigate the effect of Tor/Sch9 deficiency on acetic acid utilization, we studied the role of Ach1, a mitochondrial CoA transferase that catalyzes the CoA-SH transfer from succinyl-CoA to acetate with minor acetyl-CoA-hydrolase activity (Fig. 3A) (Fleck & Brock, 2009). The \textit{ACH1} deletion mutants and wild-type cells displayed a similar mean lifespan, although the maximum lifespan of \textit{ach1}\textsuperscript{Δ} was shorter (Fig. 5A). The \textit{sch9}\textsuperscript{Δ} \textit{ach1}\textsuperscript{Δ} double mutations caused a reversal of the longevity effect of \textit{sch9}\textsuperscript{Δ} and led to a significant increase in ethanol and acetate accumulation compared to \textit{sch9}\textsuperscript{Δ} mutants (Fig. 5A–C). The acetate in the \textit{sch9}\textsuperscript{Δ} \textit{ach1}\textsuperscript{Δ} culture reached a concentration of over 100 mM (Fig. 2A). These results indicate...
that the activation of acetic acid utilization in cells deficient in Tor/Sch9 activity depends on ACH1, whose activity is required for lifespan extension in sch9Δ.

Levels of nonfermentable carbon sources match respiration pattern in sch9Δ mutants

Down-regulation of the Tor1-Sch9 signaling pathway has been shown to induce an early increase in respiration rates (Bonawitz et al., 2007; Wei et al., 2009; Pan et al., 2011) as well as an early depletion in ethanol and acetic acid during chronological aging (Fig. 1A, B). We hypothesized that inhibition of Tor/Sch9 signaling increases respiration by promoting the catabolism of ethanol and acetic acid. Oxygen consumption measurements revealed that Sch9 deficiency induced a high rate of respiration at early stages of survival, but low respiratory rates by day 7 (Fig. 5D). In the BY4741 genetic background, deletion of SCH9 exerted a similar but delayed effect on O2 consumption (Figure S3C Supporting Information). These metabolic changes matched the pattern of ethanol and acetic acid depletion during chronological aging. However, sch9Δ mutants remained long-lived when ethanol and acetic acid were replenished every other day. Chronological survival and O2 consumption were monitored for the entire chronological lifespan study. Data are presented as mean ± SEM (n = 7). (G) WT and deletion mutants were switched to 40 mM MES buffer (pH 3.7) containing 25 mM acetic acid. Acetate utilization was monitored.
Supporting Information). Therefore, deficiencies in the Tor/Sch9 pathways activate an alternative metabolic mode that promotes rapid catabolism of ethanol, which induces high respiratory rates and a rapid catabolism of acetic acid.

To understand how long-lived mutants utilize acetic acid, we treated wild-type and sch9A mutant cells with NaCN (0.25 mM), a respiration inhibitor, on day 1 or day 3. Treatment of sch9A mutants with NaCN every other day until day 7 delayed the utilization of acetic acid (Figure S3D Supporting Information). We also employed mutants with impaired respiratory function. Coq genes catalyze ubiquinone (coenzyme Q) biosynthesis, which serves to transport electrons between the respiratory enzyme complexes I, II, and III in the mitochondrial inner membrane. Deletion of COQ2 or COQ4 genes in wild-type cells and sch9A mutants led to impaired ethanol consumption and low acetic acid accumulation during chronological aging (Figure S3E,F Supporting Information). These results indicate that acetic acid catabolism in sch9A requires a fully functional respiratory chain, but does not necessarily result in elevated oxygen consumption in the absence of ethanol. Thus, Tor/Sch9 deficiency activates an alternative metabolic acetic acid catabolism mode that does not require high oxygen consumption.

Deletion of SCH9 promotes storage of energy reserves

To determine whether acetic acid may contribute to other cellular functions not related to energy production, we measured stored nutrients. Storage carbohydrates, mainly glycogen and trehalose, serve as energy sources during stationary phase in yeast. Mutants unable to store or utilize the storage carbohydrates have significantly shortened CLS (Favre et al., 2008). We measured the glycogen and trehalose contents of cells aging chronologically. The levels of trehalose and glycogen decreased with age and reached very low levels in wild-type cells but were not depleted, even in very old organisms (Fig. 6). The sch9A mutants stored more than 2-fold higher glycogen and trehalose levels compared to wild-type cells on day 1 (Fig. 6A,B). A positive correlation between carbohydrate storage and cell survival has been shown previously (Ocampo et al., 2012). Glycogen does not appear to be as important because by day 5 wild-type cells and sch9A reached similar levels of this reserve nutrient (Fig. 6B). This suggests that the high level of trehalose may contribute to cellular protection in tor1Δ and sch9A mutants. We then treated cells with acetic acid or ethanol to determine whether these carbon sources can increase the accumulation of reserve carbohydrates. Interestingly, ethanol and acetic acid had different effects on the storage of carbon reserves. In sch9A mutants, acetic acid increased the accumulation of trehalose, while ethanol promoted its utilization. In contrast, ethanol, but not acetic acid, increased glycogen content (Fig. 6C,D). Therefore, ethanol and acetic acid trigger different metabolic pathways leading, respectively, to respiration and glycogen or trehalose storage.

Because Ach1 seems to be required for the gluconeogenic conversion of acetic acid to trehalose, we evaluated intracellular concentration of trehalose in sch9A, ach1A double knockout cells and, as predicted, its levels are significantly reduced in comparison to those in sch9A mutants (Fig. 6E). To further strengthen our observations, we evaluated trehalose concentration in chronologically aged wild-type cells that are limited in leucine, as well as bat1Δ bat2Δ double mutant cells incubated in standard SDC. As hypothesized, wild-type cells cultured in 1X leucine had a lower accumulation of trehalose than that measured for cells in SDC media (Fig. 6E). Evaluation of chronologically aged bat1Δ bat2Δ mutant cells indicated that the intracellular accumulation of trehalose is not significantly different from that of the wild-type strain. We postulate that this could be in part due to pathways that are activated in the Bat1-, Bat2-deficient cells (Fig. 6E).

Discussion

The effect of energy intake on lifespan is well documented in organisms ranging from bacteria to mice and other mammals. In fact, a 20–40% restriction in calories intake extends lifespan and reduces the incidence of a variety of diseases (Wei et al., 2008; Fontana et al., 2010). Because high levels of acetic acid have been shown to cause apoptosis in yeast (Ludovico et al., 2001), the observation that acetic acid accumulates in cultures of nondividing yeast led to the hypothesis that acetic acid plays a key role in yeast chronological aging (Burtner et al., 2009; Kaeberlein, 2010). Here, we show that under the high aeration conditions, ethanol is depleted and acetic acid accumulates reaching the toxic range associated with acute cell death, whereas under the standard aeration conditions used in our laboratory, ethanol and acetic acid represent the major carbon sources in the medium, with ethanol being the major pro-aging macronutrient. We also show that, analogously to the ketone bodies in mammals, acetic acid accumulates during the early periods of glucose depletion and can be generated from leucine catabolism in yeast cultures. Whereas wild-type cells and long-lived Ras pathway-deficient strains accumulate high levels of acetic acid, Tor/Sch9-deficient mutants rapidly catabolize it by an ACH1- and electron transport-dependent process that is required for lifespan extension and result in the storage of the protective reserve carbohydrate trehalose.

Relatively small differences in the methods used to measure lifespan may have pronounced effects on the rate of aging and cell death as well as the interpretation of the results (Gems & Partridge, 2012). We have shown that glucose and ethanol promoted aging and that medium acidification accelerated this process (Fabrizio et al., 2005). Furthermore, the calorie-restricted condition achieved by removing ethanol was sufficient to extend CLS (Fabrizio et al., 2005; Wei et al., 2009), analogously to the effect of reduction in glucose levels on both RLS and CLS. Burtner et al., on the contrary, proposed that it was the accumulation of acetic acid and not ethanol that limited yeast longevity and proposed that acetic acid may limit CLS not by promoting aging but by causing acute toxicity (Burtner et al., 2009; Kaeberlein, 2010). This difference, which appears to arise in part from the high aeration generated by experimental conditions adopted by Burtner et al., appears to be the result of an early depletion of ethanol and the accumulation of levels of acetic acid associated with cytotoxicity. Thus, to identify genes and pathways that are in fact affecting aging, it is important to use multiple experimental approaches that eliminate artifacts that may not directly impact aging (Longo et al., 2012).

Ketone bodies are the by-products of fatty acid catabolism in the liver and serve as a major source of energy during periods of food deprivation. The level of ketone bodies, normally very low in the plasma, increases when the glycogen becomes depleted and blood glucose level is low (Cahill, 2006). In yeast, acetate is produced by glucose metabolism, converted from acetaldehyde and acetyl-CoA. Here, we describe a number of parallels between yeast acetic acid and mammalian ketone bodies: (i) they are generated at high levels during periods of external glucose and glycogen depletion, (ii) leucine catabolism promotes the accumulation of ketone bodies in mammals and acetate in yeast (Bixel & Hamprecht, 1995), (iii) as for ketone bodies, yeast acetate can be transformed into acetyl-CoA which enters the TCA cycle for energy generation. The leucine metabolism pathway in yeast remains elusive. One previous study of leucine catabolism in yeast did not find
acetate as a by-product, possibly because the experiments were not performed under glucose-limited conditions (Dickinson, 2000).

Sch9 and Tor1 deficiencies have been shown to extend lifespan, increase stress resistance, and promote genomic stability in S. cerevisiae (Fabrizio et al., 2001; Wei et al., 2009). Here, we report that deletion of SCH9 causes acetate utilization in an ACH1- and electron transport chain-dependent manner. However, in sch9Δ mutants, acetic acid did not increase respiration as ethanol did, suggesting that Tor-S6K deficiency leads to a metabolic switch in which cells are able to utilize a ketone body-like metabolite for a respiration-independent purpose. Although it is not clear whether acetic acid utilization can result in ATP generation, we show that it promotes the accumulation of the stress resistance-related reserve carbon source trehalose, probably by a mechanism that requires an intact electron transport, as indicated by others (Filipak et al., 1992). In fact, it has been shown that petite cells lacking mitochondrial DNA stopped synthesizing trehalose as soon as exogenous glucose was consumed, while wild-type cells continued to accumulate the disaccharide (Enjalbert et al., 2000). A recent study suggests that the respiration thresholds are crucial for the extended CLS caused by CR and that trehalose was sufficient to restore normal CLS in respiration-deficient cells (Ocampo et al., 2012). Consistent with findings by others (Bonawitz et al., 2007; Pan et al., 2011), we demonstrate that down-regulation of the Tor/Sch9 signaling pathway induces an early increase in respiration rates during CLS and show that this is accompanied by early depletion of ethanol and acetic acid (Fig. 1A,B) as well as storage of trehalose (Fig. 6). These observations are in agreement with the findings that the effect of reduced TORC1 activity on life span in yeast may require an increased generation of ROS during growth phase. Thus, deficiency in Tor/Sch9 signaling may promote trehalose accumulation as part of a metabolic change that allows cells to catabolize acetic acid and survive under hypoxia or anoxia.

In agreement with our results, an early increase in respiration has been shown for Tor-deficient mutants and has been proposed to be required for lifespan extension (Bonawitz et al., 2007; Pan et al., 2011).

Here, we show that this early increase in respiration in Tor/Sch9-deficient cells is followed by a decrease in respiration after ethanol and acetic acid are largely depleted, indicating that the increased metabolism is reflecting the need to rapidly deplete extracellular carbon sources. However, carbon source depletion and the following hypometabolism do not fully explain the effects of Tor/Sch9 deficiency on lifespan, because tor1Δ and sch9Δ mutants display extended lifespan when carbon sources and respiration are maintained high and constant (Fig. 5E) (Wei et al., 2008). Similar to our findings, it has been reported that CR results in a 30% higher respiration rate in the growth phase and a decrease in respiration in the postdiauxic shift/stationary phase (Ocampo et al., 2012). This finding suggests that CR and reduced Tor/Sch9 signaling may regulate common genes, which first promote respiration and depletion of nonfermentable carbon sources and then cause a hypometabolic mode associated with lifespan extension (Wei et al., 2009). In agreement with protective effects of β-hydroxybutyrate demonstrated in mammals (Shimazu et al., 2013).

In summary, our study suggests that acetic acid may represent a ketone body-like carbon source generated during periods of starvation and utilized by Tor/Sch9-deficient cells through an Ach1- and electron transport chain-dependent mechanism.
transport-dependent pathway which promotes the accumulation of the protective carbohydrate trehalose and lifespan extension.

Experimental procedures

Yeast strains

Most of the Saccharomyces cerevisiae strains used in this study were derived from DBY746 (MATa, leu2-3, 112, his3A1, trp1-289, ural3-52, Gal4+). Some experiments were also confirmed in the BY4741 (MATa, his3A1, leu2Δ0, met15Δ0, ural3Δ0) genetic background. Knockout strains were generated by one-step gene replacement (Longtine et al., 1998). The sch9A, cyr1::mTn, and ras2Δ mutants have been described previously (Fabrizio et al., 2001; Wei et al., 2008). Strains with SDC-LacZ reporter gene were generated by integrating into the URA3 locus the pMM2 plasmid which contains four tandem repeats of STRE motif from the HSP12 sequence (−221 to −241) fused with the LacZ-coding sequence (Boy-Marcotte et al., 1998). The plasmid pCDV454 containing LacZ reporter under the control of a 37-bp SSA3-PDS region (−206 to −170) (Pedruzzi et al., 2000) was integrated into the URA3 locus of wild-type cells to generate PDS-LacZ gene reporter strain. The protocol of reporter gene activity measurement has been described elsewhere (Wei et al., 2008).

Growth conditions

Yeast cells were grown in SCD containing 2% or 0.5% glucose supplemented with a 4-fold excess of tryptophan, leucine, histidine, and uracil to avoid possible lifespan modification due to auxotrophic deficiencies of the strains. Standard SDC medium without excess of essential amino acids was used for comparison. Day 3 wild-type cultures were centrifuged, and filtered supernatants were used to perform medium switch experiments.

Chronological lifespan assay

Yeast chronological lifespan was measured as previously described (Fabrizio and Longo, 2003). Overnight SDC culture was diluted (OD 0.1) into fresh SDC medium (with 5:1 flask to culture volume) and incubated at 30 °C with shaking (200 rpm). 24 h later was considered as day 1. Every 48 h, properly diluted culture was plated onto YPD plates and then incubated at 30 °C for 2–3 days. Chronological lifespan was assessed by colony-forming units (CFUs) measurement. Day 3 CFUs were considered as the initial survival (100%) and used to determine the age-dependent mortality. Mean lifespan was calculated from curve fitting of the survival data with the statistical software Prism (GraphPad Software, Inc., La Jolla, CA, USA). For medium switch experiments, cells were grown in SDC for 3 days, washed twice with sterile distilled water, and suspended in MES buffer (40 mM, pH 3.7 or pH 6.0) with different carbon sources or other media as stated.

Stress resistance assay

Heat shock resistance was measured by spotting serial dilutions (5-fold or 10-fold dilution) of cells removed from cell cultures onto YPD plates and incubating at 55 °C (heat-shocked) and at 30 °C (control) for 60–150 min. After the heat shock, plates were transferred to 30 °C and incubated for 2 days. For oxidative stress resistance assays, cells were diluted 10-fold in K-phosphate buffer (pH 6.0) and treated with 70–300 mM H2O2 for 30 min. Serial dilutions (5-fold or 10-fold) of untreated control and H2O2-treated cells were spotted onto YPD plates and incubated at 30 °C for 2–3 days.

LacZ reporter gene assay

1 mL culture was collected, washed once with water, pelleted, and then stored at −80 °C. 100 μl of low-salt lysis buffer (50 mM Tris, pH 7.5, 0.1% (v/v) Triton X-100, 1X protease inhibitor, 100 mM NaCl, 2 mM EDTA, 2 mM EGTA, 50 mM NaF) and an equal volume of glass beads were added to the cell pellets. The mixture was vortexed at maximum speed for 45 s and then chilled on ice for 45 s. This was repeated five times. The mix was centrifuged at 14 000 rpm at 4 °C for 5 min and the supernatant was transferred to a clean tube. Protein concentration of the lysate was assayed with a BCA kit (Pierce). 55 μl of lysis (or appropriately diluted samples) was mixed with 85 μl of substrate solution (1.1 mg mL−1 ONPG in Z buffer: 60 mM Na2HPO4, 40 mM NaH2PO4, 10 mM KCl, 1 mM MgSO4, 50 mM 2-mercaptoethanol, pH 7.0). Absorbance at 420 nm was read every 5 min until 30 min after the reaction was started. LacZ activities were determined by fitting the A420/time data to that of serial-diluted recombinant β-galactosidase (Promega) and were further normalized with total protein.

Acetic acid and ethanol measurements

Yeast chronological aging cultures were centrifuged, and supernatants were collected and frozen at −80 °C. Acetic acid and ethanol concentrations of the conditioned medium were determined by acetic acid and ethanol assay kits (R-biopham Cat. 10148261035 and 10176290035) following the manufacturer’s recommended protocol.

Oxygen consumption measurement

Oxygen consumption of 2 mL culture was measured in a glass container magnetically stirred to avoid cell precipitation at 30 °C in a water bath using a Clark-type electrode. According to the manufacturer, the liquid culture was assumed to contain the same amount of oxygen as water equilibrated with 21% oxygen in 1 atmosphere pressure (5.02 μl mL−1). Oxygen level in the culture was automatically recorded every 5 s until a trace of straight line was obtained, which suggested that the oxygen consumption had reached a steady state. The amount of oxygen consumed was further normalized by CFU.

Quantification of glycogen and trehalose

2.5–3 mL cell cultures was collected from indicated cultures at indicated time points. Cells were washed with water, pelleted, and stored at −80 °C until measurement. The cell pellet weights were measured at the time of collection for normalization purpose. Intracellular glycogen and trehalose contents were determined as previously described (Parrou & Francois, 1997). Briefly, cell pellets were resuspended in 250 μl of 0.25 mM Na2CO3 and incubated at 95 °C with occasional stirring for 4 h. Subsequently, the suspension was buffered to pH 5.2 by adding 150 μl of 1 M acetic acid and 600 μl of 0.2 M NaAc (pH 5.2). For the trehalose content determination, half of the mixture was incubated overnight at 37 °C in the presence of 3 mg trehalase (Sigma, USA) under constant agitation. For the glycogen content determination, the second half was digested overnight at 57 °C with continuous shaking on a rotary shaker in the presence of 100 μg α-amylglucosidase (Sigma-Aldrich, St. Louis, MO, USA). Finally, mixture was centrifuged and the supernatant was collected. Glucose released from trehalose and glycogen digestion was
quantified using the Glucose Assay Kit (R-biopham, Cayman Chemical Company, Ann Arbor, MI, USA) following the manufacturer’s protocol.

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Conflict of interest

None declared.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher’s web-site.

Fig. S1 (A, B) Cells were grown in 2% glucose SDC medium until day 2.

Fig. S2 (A, B) Wild-type (DBY746) growing in 2% glucose SDC with 4X or 1X HIS, URA, LEU, TRP, or 1X LEU with 4X IS, URA, TRP.

Fig. S3 (A, B) Wild-type and sch9Δ mutant cells grown in 2% glucose SDC until day 3 and then washed 3 times and switched to 40 mM MES buffer (pH 3.7) or buffer with physiological levels of ethanol (0.8%) or acetic acid (50 mM).

Fig. S4 (A) Chronological survival of wild-type cells in media limited in leucine.