Glucose feeds the tricarboxylic acid cycle via excreted ethanol in fermenting yeast

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Ethanol and lactate are typical waste products of glucose fermentation. In mammals, glucose is catabolized by glycolysis into circulating lactate, which is broadly used throughout the body as a carbohydrate fuel. Individual cells can both uptake and excrete lactate, uncoupling glycolysis from glucose oxidation. Here we show that similar uncoupling occurs in budding yeast batch cultures of *Saccharomyces cerevisiae* and *Issatchenkia orientalis*. Even in fermenting *S. cerevisiae* that is net releasing ethanol, media 13C-ethanol rapidly enters and is oxidized to acetaldehyde and acetyl-CoA. This is evident in exogenous ethanol being a major source of both cytosolic and mitochondrial acetyl units. 2H-tracing reveals that ethanol is also a major source of both NADH and NADPH high-energy electrons, and this role is augmented under oxidative stress conditions. Thus, uncoupling of glycolysis from the oxidation of glucose-derived carbon via rapidly reversible reactions is a conserved feature of eukaryotic metabolism.

Fermentation occurs widely across kingdoms, converting glucose into organic waste products. In mammals, the main such product is lactate. Until recently, it was commonly assumed that the liver and kidney were special in their capacity to clear circulating lactate, reconverting the waste (lactate) into fuel (glucose). New evidence suggests, however, that most mammalian tissues take up circulating lactate and oxidize it via the tricarboxylic acid (TCA) cycle. Indeed, it seems that most carbohydrate oxidation in mammals, rather than occurring by a tissue taking up glucose and fully oxidizing it to carbon dioxide, instead involves carbon flow through circulating lactate as a metabolic intermediate. Thus, glycolysis is uncoupled from the TCA cycle via cellular uptake and/or excretion of lactate. Biochemically, this occurs through the rapidly reversible reactions linking intracellular pyruvate, via lactate dehydrogenase and monocarboxylate transporters, to circulating lactate.

Baker’s yeast (*S. cerevisiae*) is a prototypical fermentative unicellular organism. Its rapid catabolism of glucose into ethanol + CO₂ plays a central role in human society, contributing to such diverse fields as baking, beverages and biofuels. *S. cerevisiae* is capable of growing aerobically on substrates including galactose, glycerol and ethanol. But when provided with ample glucose, it will ferment even in the presence of adequate oxygen, a phenomenon known as the Crabtree effect. When glucose runs out, after a delay to rewire metabolism, aerobic growth will resume (the diauxic shift). Some other budding yeasts, such as *Issatchenkia orientalis*, engage in extensive oxidative metabolism even when glucose is present (that is, are Crabtree negative) and do not show a diauxic shift but, nevertheless, secrete some ethanol. As glucose fermentation in yeast parallels aerobic glycolysis in mammals, we were curious whether it similarly involves reversible excretion and uptake of the ‘waste product’ (ethanol) rather than distinct phases of waste production and subsequent consumption. We further wondered whether any such ethanol uptake during net fermentative metabolism might contribute to yeast’s metabolic robustness.

Understanding these questions is relevant both for basic science and for bioengineering, with ethanol uptake undesirable in yeast deployed for producing ethanol as biofuel. With these motivations in mind, we show that, even when fermenting, yeasts actively exchange environmental ethanol for intracellular acetalddehyde at a sufficiently rapid rate that intracellular acetyl units come substantially from environmental ethanol, in addition to directly from glucose. Moreover, such exchange enables ethanol to be a major source of NADH and NADPH, especially under oxidative stress conditions.

Results

Fermenting baker’s yeast assimilates environmental ethanol. Can then enter and exit cells via simple diffusion. Thus, exogenous ethanol may enter yeast, even if they are simultaneously excreting ethanol made internally from glucose. To differentiate two-carbon (2C) units from environmental ethanol versus internal glucose catabolism, we grew yeasts in typical minimal media (yeast nitrogen base, aerated, 30 °C) with unlabeled glucose until mid-exponential phase. We then pelleted the cells and resuspended them in yeast nitrogen base containing both glucose and ethanol, whose isotopic composition was under experimental control. The glucose and ethanol concentrations in the resuspension media were selected to approximate those naturally occurring during mid-exponential *S. cerevisiae* growth in yeast nitrogen base with glucose as the carbon source (recognizing that by mid-exponential phase yeast will have converted a substantial amount of glucose into ethanol). Specifically, we provided glucose and ethanol at either equimolar concentrations (42 mM each, ‘equimolar’) or a 1:1 mixture based on the number of carbon atoms (28 mM glucose and 84 mM ethanol, ‘equicarbon’) (Fig. 1a).

We used ¹³C NMR to measure rates of glucose uptake (f_{glc_up}) and conversion to environmental ethanol via pyruvate decarboxylase (f_{PCDh_up}) from the *S. cerevisiae* cultures with [U-¹³C]glucose and unlabeled ethanol (Fig. 1b and Extended Data Fig. 1). The rates measured by ¹³C NMR (f_{glc_up} and f_{PCDh_up}) are similar among...
strains of *S. cerevisiae* with different respiratory capacity (FY4 and CEN.PK) and media substrate ratios (equimolar or equicarbon) (Extended Data Fig. 1a,b). In parallel, net ethanol flux was measured by 1H NMR, revealing active fermentation (that is, net ethanol excretion) (Fig. 1c).

The yeast ethanol assimilation pathway involves oxidation of ethanol to acetate, which is converted into cytosolic acetyl-CoA by acetyl-CoA synthetases. To trace potential ethanol uptake and use, we directly measured cellular acetyl-CoA labeling distributions by liquid chromatography–mass spectrometry (LC–MS) in *S. cerevisiae*.
grown with unlabeled glucose and 13C-labeled ethanol (Fig. 1d), finding substantial labeling (more than 50%) from environmental ethanol (Fig. 1e). This high [M + 2] labeled fraction of acetyl-CoA is a steady-state measurement (Extended Data Fig. 1c) and is consistent across strains and media compositions (Extended Data Fig. 1d). In these fermenting cells, ethanol carbon did not enter glycolytic intermediates, as shown by the absence of [M + 2] labeling, consistent with gluconeogenesis being inactive (Extended Data Fig. 2).

We built a 13C metabolic flux model to estimate the reversibility of the ethanol assimilation pathway (Fig. 1f). The model was constrained by the measured glucose uptake rate, the net ethanol excretion rate, PDC flux (the flux representing gross glucose conversion to ethanol) and acetyl-CoA labeling from [U-13C]ethanol. The model confirmed low PDH and high PDC flux, as typical for fermenting S. cerevisiae (Fig. 1g). Notably, it revealed a fast exchange flux between ethanol and acetaldehyde, with ethanol a major source of acetaldehyde even though net flux is in the direction of ethanol excretion (Fig. 1g). This rapid exchange flux explains the substantial acetyl-CoA labeling from environmental ethanol (Fig. 1e).

Environmental ethanol contributes to fatty acid synthesis. Acetyl-CoA exists as discrete cytosolic and mitochondrial pools. Fatty acid synthesis uses cytosolic acetyl-CoA (Fig. 2a); thus, fatty acid labeling selectively represents cytosolic acetyl-CoA labeling. In S. cerevisiae fed either the equimolar or equicarbon mixture of unlabeled glucose and [U-13C]ethanol, most of the carbon in the newly synthesized fatty acids (that is, those containing at least some label) was from environmental ethanol (Fig. 2b and Extended Data Fig. 3).

To quantify the fraction of cytosolic acetyl-CoA coming from environmental ethanol, we fit the observed fatty acid mass isotope distribution to a binomial, reflecting the fact that each 2C unit incorporated into fat is selected stochastically, with the assumption that only labeled fatty acids are newly synthesized. The simple binomial fit well, consistent with a homogeneous environmental ethanol contribution across different cells in the population of around 60% of lipogenic acetyl-CoA (Fig. 2c). Thus, rather than being derived mainly internally by glycolysis and subsequent pyruvate catabolism, when environmental ethanol is present, cytosolic acetyl-CoA in baker’s yeast comes also from ethanol.

Environmental ethanol supplies mitochondrial acetyl-CoA. Formation of cytosolic acetyl-CoA from acetate is catalyzed by acetyl-CoA synthetases12.14. Such synthetases are not known in yeast mitochondria. Accordingly, we were curious whether environmental ethanol could also contribute to mitochondrial acetyl-CoA. To this end, using the same tracing strategy as above, we examined whether environmental ethanol would label a metabolic product that is produced mitochondrially from acetyl-CoA, N-acetylglutamate (NAG), an intermediate in the arginine biosynthesis pathway (Fig. 2d). In S. cerevisiae fed either equimolar or equicarbon unlabeled glucose and [U-13C]ethanol, NAG was labeled both [M + 2] and [M + 4], whereas glutamate was only labeled [M + 2] (Fig. 2e and Extended Data Fig. 4). To quantitate the fraction of mitochondrial acetyl-CoA coming from environmental ethanol, we inferred mitochondrial acetyl-CoA labeling from the observed mass isotope distribution of NAG and glutamate. The calculated [M + 2] fraction of mitochondrial acetyl-CoA is around 60% (Fig. 2f), similar to cytosolic acetyl-CoA. Thus, environmental ethanol is a major source of both cytosolic and mitochondrial acetyl-CoA.

The enzyme succinyl-CoA acetate CoA transferase (Ach1) has been proposed as a potential means of generating mitochondrial acetyl-CoA from acetate in S. cerevisiae, but its physiological role has remained unproven15. Ach1 deletion completely abolished [M + 4] NAG (Fig. 2e and Extended Data Fig. 4a,b), with the inferred mitochondrial acetyl-CoA labeling zero in this deletion strain (Fig. 2f). Notably, Δach1 nevertheless has similar whole cell [M + 2] acetyl-CoA labeling from ethanol (Extended Data Fig. 4c,d), implying that only a small fraction of total cellular acetyl-CoA is mitochondrial, with Ach1 the key mitochondrial acetyl-CoA labeling enzyme.

TCA intermediates are made from environmental ethanol. Acetyl-CoA contributes to the TCA cycle via citrate synthase (Fig. 3a). From [U-13C]ethanol tracing in fermenting S. cerevisiae, we observed [M + 2] (iso)citrate, aconitate, α-ketoglutarate and succinate. Fumarate, malate and aspartate (whose carbon skeleton comes from oxaloacetate) remained, however, largely unlabeled (Fig. 3b and Extended Data Fig. 5). The extensive labeling of succinate with limited labeling of fumarate or oxaloacetate pinpoints succinate dehydrogenase (complex II in the electron transport chain) as being functionally blocked during fermentative growth of baker’s yeast16. Instead of being made by TCA turning, oxaloacetate and malate are generated by pyruvate carboxylase, using pyruvate made from glucose. Nevertheless, acetate from environmental ethanol is assimilated into the TCA cycle and drives conversion of these four-carbon TCA intermediates into citrate, α-ketoglutarate and α-ketoglutarate’s amino acid products.

To explore the generality of ethanol’s contributing to acetyl-CoA and, thereby, TCA intermediates in glucose-fed budding yeast, we carried out analogous experiments in I. orientalis, Crabtree-negative yeast diverged from S. cerevisiae roughly 200 million years ago. In both equimolar and equicarbon conditions, [U-13C]ethanol generated [M + 2] labeled TCA intermediates to a similar extent in both yeast species (Fig. 3b,c). In I. orientalis, we also observed more heavily labeled TCA intermediates indicative of active ethanol assimilation and full TCA turning (Extended Data Fig. 5). Thus, assimilation of environmental ethanol in the presence of glucose is a feature of both Crabtree-positive and Crabtree-negative budding yeasts.

Concentration dependence of ethanol use. Although reflecting the levels of ethanol typically found in dense fermenting yeast cultures, the equimolar and equicarbon conditions (28 mM and 42 mM ethanol, respectively) contain more ethanol than is found in many physiological environments. For example, early log-phase growth of S. cerevisiae in glucose batch culture (initial optical density of 0.1 OD grown for 1 hour) generates ethanol concentrations around 0.5 mM17-19 (Fig. 1c). At this concentration, ethanol accounts for less than 1% of carbon in the media (>99% being glucose). Nevertheless, the 13C-ethanol contributes discernibly to the cellular acetyl-CoA in S. cerevisiae and is a major source in I. orientalis (Fig. 3d,e). At 5 mM concentration, ethanol becomes a major acetyl-CoA source also in S. cerevisiae. Thus, the importance of environmental ethanol as an acetyl-CoA source depends on concentration and is substantial at ethanol levels found in moderate to dense glucose-fed yeast cultures (Extended Data Fig. 6).

Acetaldehyde oxidation feeds NADPH. Ethanol re-oxidation has redox consequences. Ethanol conversion to acetaldehyde generates NADH. Further oxidation of acetaldehyde into acetate via aldehyde dehydrogenase generates NADPH. When the canonical main NADPH production pathway, the oxidative pentose phosphate pathway, is deleted, the acetaldehyde dehydrogenase Ald6 is essential for yeast growth19.

To measure redox cofactor contributions from environmental ethanol, we transferred fermenting S. cerevisiae into glucose ethanol as above, with either the glucose or the ethanol deuterated at position 1. Specifically, we compared NADH and NADPH labeling from [1-2H]glucose (the labeled hydrogen is transferred to NADPH via G6PD, encoded by gene zwf1) and [1,1-2H2]ethanol (the labeled hydrogen is transferred to NADH by ADH and to NADPH via Ald). Direct measurement of 2H-labeling in NADH and NADPH is technically challenging due to limited abundance and stability, but,
Fig. 2 | Environmental ethanol contributes to both cytosolic and mitochondrial acetyl-CoA in fermenting S. cerevisiae. a, When fed with labeled ethanol, the resulting $^{13}$C-labeled acetyl-CoA is incorporated into newly synthesized fatty acids. As both labeled and unlabeled cytosolic acetyl-CoA are randomly incorporated into growing fatty acid chains, the resulting fatty acid mass isotope distribution follows a binomial probability distribution. b, Fatty acid labeling (C16:0) pattern from equimolar glucose: $^{13}$C-ethanol co-feeding experiment as in Fig. 1d (mean, s.e., n = 3 biological replicates). In brief, newly synthesized fatty acids are getting labeled by [M + 2] acetyl-CoA, which is a result of $^{13}$C-ethanol uptake from growth media by S. cerevisiae. c, Cytosolic acetyl-CoA labeling fitted from fatty acid labeling from equimolar glucose: $^{13}$C-ethanol co-feeding experiment as in Fig. 1d (mean, s.e., n = 6, three biological replicates with results from both C16:0 and C18:0) and whole-cell data from Fig. 1e (mean, s.e., n = 3 biological replicates). d, Synthesis of the arginine precursor N-acetylg glutamate (NAG) in S. cerevisiae takes place in mitochondria (created with BioRender). A linear algebra deconvolution of the labeling fractions of glutamate and NAG can compute the mitochondrial acetyl-CoA labeling. e, Glutamate (Glu) and NAG labeling from the glucose: $^{13}$C-ethanol co-feeding experiment as in Fig. 1d, including also data for Δach1 yeast (thereby identifying Ach1 as an enzyme essential for mitochondrial assimilation of ethanol-derived carbon into acetyl-CoA) (mean, s.e., n = 3 biological replicates; ***P < 0.001 by two-sided t-test). In brief, given the observed highest isotopic label of Glu is [M + 2], if mitochondrial acetyl-CoA labeled [M + 2] by $^{13}$C-ethanol is uptaken by S. cerevisiae from growth media, newly synthesized NAG will be in part [M + 4]. f, Mitochondrial acetyl-CoA [M + 2] fraction fitted from glutamate and NAG labeling in e (mean, s.e., n = 3 biological replicates). WT, wild-type.
Fig. 3 | Carbons from ethanol feed into TCA intermediates. a, Schematic of TCA cycle highlighting observed TCA labeling from glucose: $^{13}$C-ethanol co-feeding as in Fig. 1d, where [(M + 2)] acetyl-CoA is a result of uptaking $^{13}$C-ethanol from growth media by S. cerevisiae (created with BioRender). $^{13}$C is in green, and $^{12}$C is in white. b, Labeling patterns of TCA intermediates for S. cerevisiae FY4 in equimolar glucose: $^{13}$C-ethanol (mean, s.e., n = 3 biological replicates). c, As in b for I. orientalis SD108. d, Concentration dependence of environmental ethanol contribution to acetyl-CoA in S. cerevisiae grown in standard glucose-rich (1% w/v) minimal media to OD = 0.1, spiked with 0.05, 0.5 or 5 mM $[^{13}$C]ethanol and harvested 1 hour later (mean, s.e., n = 3 biological replicates). e, As in d for I. orientalis.
nevertheless, we observed clear isotope shifts upon exposure to the 1H-labeled substrates, confirming contribution from glucose’s position 1 hydrogen to NADPH and from ethanol’s position 1 hydrogen to both NADH and NADPH (Extended Data Fig. 7a–f).

To obtain more precise and compartment-specific information, we used fatty acid labeling to read out cytosolic NADPH labeling. Fatty acid synthesis incorporates two NADPH hydrides per acetyl group (Fig. 4a)49. Strikingly, we observed greater deuterium labeling of fatty acids from [1-1H2]ethanol than from [1-2H]glucose (Extended Data Fig. 8). This reflects a major contribution of acetaldehyde to cytosolic NADPH via Ald6, of yet greater magnitude than the contribution of glucose-6-phosphate to cytosolic NADPH via the oxPPP (Fig. 4b,c).

To convert the observed labeling into quantitative contributions to NADPH, we need to account for deuterium loss from NADPH via hydrogen–deuterium exchange with water mediated by flavin enzymes48,49. Experiments culturing cells in D2O revealed that about half of cytosolic NADPH hydrogen nuclei come from water via hydrogen–deuterium exchange. Such exchange does not account for any NADPH’s high-energy electrons but merely dilutes deuterium tracer signal from the actual hydride donors such as [1-1H]glucose or [1-2H]acetate (Extended Data Fig. 7g–i). Correcting for such exchange (and for the extent of substrate labeling), we observed that the oxPPP and Ald6 together account for most cytosolic NADPH, with the contribution of ethanol via Ald6 roughly double that of glucose via the oxidative pentose phosphate pathway (Fig. 4d).

Consistent with ethanol oxidation and oxPPP being alternative cytosolic NADPH production pathways, in Δald6, oxPPP contribution to NADPH production (based on fatty acid labeling patterns) is nearly twice as high as in wild-type (Fig. 4c and Extended Data Figs. 8a,b and 9). In Δzwf1, Ald6 contribution to NADPH production (based on fatty acid labeling patterns) similarly doubles (Fig. 4b and Extended Data Figs. 8c,d and 9). Thus, ethanol is an important source of both acetyl and hydride units in baker’s yeast.

**Ethanol becomes a greater NAD(P)H source upon H2O2 stress.** We were curious whether *S. cerevisiae* cells might shift between glucose or ethanol as NAD(P)H sources in response to environmental conditions. To explore this possibility, we grew yeast in glucose:ethanol with one substrate 1H-labeled as above, spiked in H2O2 to a final concentration of 20 mM, and rapidly sampled metabolites and their labeling11,12 (Fig. 5a). Upon adding H2O2, the NADH concentration and NADH/NAD+ ratio fell markedly (Fig. 5b,c). Such a drop was expected, given that oxidative stress is known to oxidize the GADPH’s active site cysteine and, thereby, block glycolytic flux and NADH production11. Consistent with GAPDH being shut off, in addition to an increase of fructose-1,6-bisphosphate (FBP) (Extended Data Fig. 10), we observed increased NADH labeling from ethanol, which became the dominant NADH hydride source (Fig. 5d). Thus, ethanol catabolism is a crucial source for NADH when glycolysis is blocked by oxidative stress.

A classical rationale for glycolytic blockade by oxidative stress is to divert flux into the oxidative pentose phosphate pathway to help maintain NADPH homeostasis. The same concentration of hydrogen peroxide that markedly suppressed NADH had no overt effect on NADPH pool size or the NADPH/NAD+ ratio (Fig. 5e,f). However, rather than increasing the fractional oxidative pentose phosphate pathway contribution to NADPH as measured by [1-1H]glucose, this contribution was decreased, with ethanol’s fractional contribution to NADPH markedly increased (Fig. 5g). Thus, in contrast to the common assumption that the
predominant NADPH production route during oxidative stress is the oxidative pentose phosphate pathway, we observe a substantial and augmented NADPH contribution from ethanol oxidation under H₂O₂ stress²⁶,²⁴.

Discussion

A fundamental metabolic question is, ‘Which pathways are coupled versus independent?’ Here we present evidence that fermenting yeast has a predominant NADPH production route during oxidative stress is the oxidative pentose phosphate pathway, we observe a substantial and augmented NADPH contribution from ethanol oxidation under H₂O₂ stress²⁶,²⁴.

Although the net release of ethanol by fermenting yeast has been long appreciated, we are unaware of prior demonstration that fermenting yeast cultures simultaneously engage in extensive ethanol uptake. Through experiments with [⁵⁷C]-ethanol, we show that, under typical mid-exponential fermentative growth conditions, environmental ethanol, rather than mitochondrial pyruvate catabolism, supplies a majority of both cytosolic and mitochondrial acetyl-CoA. This observation aligns with prior literature finding that, when [U⁵⁷C]glucose is spiked into fermenting yeast cultures (which naturally contain ethanol), acetyl-CoA is labeled less than other central metabolites²⁶. The importance of the ethanol assimilation pathway depends on the environmental ethanol concentration, with ethanol becoming a major acetyl-CoA source at millimolar level. In mitochondria, we prove that the ethanol assimilation pathway involves the CoA-transferase Ach1²⁹–³¹. The assimilated ethanol was originally produced from glucose but, at the population level, the pathway from glycolysis to the TCA cycle (and other acetyl-CoA products such as amino acids and fatty acids) flows through pyruvate dehydrogenase and environmental ethanol.

Fig. 5 | Instead of oxPPP, ethanol oxidation provides major fraction of active hydrides in the presence of H₂O₂. a, Schematic of experimental workflow of administration of H₂O₂ oxidative stress to yeast (S. cerevisiae FY4) cultures pre-incubated with equi-carbon glucose:ethanol (created with BioRender). Temporal changes within the first 60 seconds after H₂O₂ shock are captured by rapid quenching of metabolism at time points of 15 seconds, 30 seconds and 60 seconds. b, NADH pool size (mean, s.e., n = 6 biological replicates, negative linear trend, P = .0016, ordinary one-way ANOVA). c, NADH:NAD⁺ ratio (mean, s.e., n = 6 biological replicates, negative linear trend, P = .0006, ordinary one-way ANOVA). d, NADH active hydride labeling from [1,1-²H₂]ethanol (orange) and [¹-²H]glucose (gray) (mean, s.e., n = 3 biological replicates, P < .01(*)). Increase in the NADH active hydride labeling from labeled ethanol is statistically significant (positive linear trend, P < .001, ordinary one-way ANOVA). e, NADPH pool size (mean, s.e., n = 6 biological replicates). f, NADPH:NAD⁺ ratio (mean, s.e.m., n = 6 biological replicates). g, NADPH active hydride labeling from [1,1-²H₂]ethanol (orange) and [¹-²H]glucose (gray) (mean, s.e., n = 3 biological replicates, P = .02, two-tailed paired t-test). Increase in the NADPH active hydride labeled by ethanol is statistically significant (positive linear trend, P < .001, ordinary one-way ANOVA).
Our methods cannot differentiate whether this flux through environmental ethanol involves intercellular ethanol exchange (that is, from ethanol-secreting fermentative cells to ethanol-consuming oxidative cells) or pathway reversibility at the single-cell level (that is, simultaneous ethanol secretion and re-uptake by the same cells). An intriguing possibility is that ethanol re-uptake coordinates with the yeast metabolic cycle, occurring primarily in cells in the metabolic cycle's oxidative phase, with ethanol produced by cells in the reductive building and charging phases.

Simultaneous ethanol excretion and uptake simplify regulation of the fate of pyruvate, circumventing the challenge of partitioning it optimally between fermentation and acetyl-CoA. Ethanol excretion is the default, with the resulting environmental ethanol providing a reservoir to help meet cellular two-carbon unit demands. Such a reservoir is not essential, as yeast can grow rapidly in dilute cultures where ethanol is scarce or in contexts where excreted ethanol is diluted away by fluid flow. But when available, environmental ethanol helps assure adequate availability of both carbon and high-energy electrons even if glycolysis is impaired.

Notably, because simultaneous ethanol excretion and re-assimilation is a default state, yeast can access both the carbon and high-energy electrons from environmental ethanol without any remodeling of their internal metabolic machinery. Such access is particularly evident during acute redox stress, which impairs glycolysis through inhibitory oxidation of the central glycolytic enzyme GAPDH. Under this circumstance, ethanol becomes the predominant source of both NADH and NADPH, the latter being critical for survival of oxidative stressors. Thus, uncoupling of glycolysis from the TCA cycle via ethanol provides yeast with metabolic flexibility, decreases regulatory complexity and enhances robustness.

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References
1. Weinhouse, S., Warburg, O., Burk, D. & Schade, A. L. On respiratory impairment in cancer cells. Science 124, 267–272 (1956).
2. Wolfe, A. J. The acetate switch. Microbiol. Mol. Biol. Rev. 69, 12–50 (2005).
3. De Deken, R. H. The Crabtree effect: a regulatory system in yeast. Biochim. Biophys. Acta 1539, 75–81 (2000).
4. Bui, L. T. et al. Conservation of ethanol fermentation and its regulation in Saccharomyces cerevisiae. Biochem. Biophys. Acta 1864, 473–485 (2016).
5. Brauer, M. J., Saldanha, A. J., Dolinski, K. & Botstein, D. Homeostatic adjustment and metabolic remodeling in glucose-limited yeast cultures. Mol. Biol. Cell 16, 2503 (2005).
6. Park, J. O. et al. Metabolite concentrations, fluxes and free energies imply efficient enzyme usage. Nat. Chem. Biol. 12, 482–489 (2016).
7. Grabowska, D. & Chelstowska, A. The ALD6 gene product is indispensable for providing NADPH in yeast cells lacking glucose-6-phosphate dehydrogenase activity. J. Biol. Chem. 278, 13984–13988 (2003).
8. Stolz, D. M. & Grant, C. M. Protein S-thiolation targets glycolysis and protein synthesis in response to oxidative stress in the yeast Saccharomyces cerevisiae. Biochem. J. 374, 513 (2003).
9. Minard, K. I. & McAlister-Henn, L. Sources of NADPH in yeast vary with carbon source. J. Biol. Chem. 280, 39890–39896 (2005).
10. van Hoek, M. J. A. & Merks, R. M. H. Redox balance is key to explaining full vs. partial switching to low-yield metabolism. BMC Syst. Biol. 6, 22 (2012).
11. Vemuri, G. N., Eiteman, M. A., McEwen, J. E., Olsson, L. & Nielsen, J. The role of the HXXH domain in the regulation of the TCA cycle via ethanol provides yeast with metabolic flexibility, decreases regulatory complexity and enhances robustness.

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Materials. Yeasts were grown in yeast nitrogen base without amino acids (Sigma-Aldrich, Y0626) with carbon source added separately. BD DifcoYPD Broth (BD, 242820) was used as the media for reviving frozen cells or growing cells to be filtered. Glycerol (Sigma-Aldrich, G5516) was added at a 1:1 volume ratio to YPD yeast cultures in cryovials (Nalgene, 5000-1020, or Corning, 430289). Glucose (Sigma-Aldrich, D9434), [U-13C6]glucose (Cambridge Isotope Laboratories, CLM-1396), [13C]ethanol (Sigma-Aldrich, 427039), [1-13C]ethanol (Sigma-Aldrich, 347434), ethanol (Decon Labs, DSP-MD.43) and 1-13H-glucose (Omnicron Biochemistry, GLC-032) were used as carbon sources. Tap water filtered with Milli-Q Reference Water Purification System (Millipore Sigma, C79625) and D2O (Cambridge Isotope Laboratories, DLM-4) were used as the water source for yeast cultures. Millipore Sigma Stericup Quick Release-HV Sterile Vacuum Bottle Top Filtration Systems with 0.22-µm PES filters were used to sterilize all media for yeast cultures, and 0.45-µm nylon filters (GVs North America, 123776) were used to filter yeast for metabolite extraction. 1-[3,3,3]-propionate-2,2,3,3-d4 acid sodium salt (TMSP) (Sigma-Aldrich, 269913) was used as 1H NMR internal standard, and sodium formate-13C (Sigma-Aldrich, 279941) was used as 13C NMR internal standard. Experimental cultures were grown in 14-ml polypropylene round-bottom tubes (Falcon, 352059). HPLC vials (Thermo Fisher Scientific, 200-046, 501-331) were used for [U-13C]glucose and [U-12C]glucose and 1.17 p.p.m. (t, 3H) for ethanol. Water 3.2 p.p.m. (dd, 1H) for \( \delta_{13C} \) of [U-12C]glucose and 4.66 p.p.m. and spw1 with some modifications22. Cells were incubated in the experimental culture media containing either (1) YNB + 28 mM unlabeled glucose and 42 mM [1,1-2H2]ethanol or (2) YNB + 42 mM [U-13C]glucose and 42 mM unlabeled ethanol. The equilibration case, each portion was switched into the media containing either (1) YNB + 28 mM unlabeled glucose and 84 mM [U-13C]ethanol or (2) YNB + 28 mM [U-12C]glucose and 84 mM unlabeled ethanol. OD was taken before and after the incubation period for flux calculation. Before switching and 1 hour after the media switch, 0.5 ml of the culture media was mixed with 50 µl of the 50 mM deuterated TMSP standard solution in D2O and loaded onto NMR instruments. 1H NMR & 0 ppm (s, 9H) for the TMS standard, 3.2 p.p.m. (dd, 1H) for β-glucose and 1.17 p.p.m. (t, 3H) for ethanol. Water suppression was achieved by O2P- 4.66 p.p.m. and spw1 = 0.002. To achieve quantitative NMR, D, was set to 5 seconds and 90° pulse (p) to 11.69 µs.

3C qNMR measurement. The sample collection procedure and preparation were identical to the ‘H NMR excretion profile, except that 3C sodium formate (0.1M) was used as a standard instead of deuterated TMS. 13C NMR: 168 p.p.m. (s, 1C) for the sodium formate standard, 161 p.p.m. (d, 1C) for glucose and 57 p.p.m. (d, 1C) for ethanol. Samples of media that the cells are switched into were measured for the sodium formate standard, 161 p.p.m. (d, 1C) for glucose and 57 p.p.m. (d, 1C) for ethanol. Water suppression was achieved by O2P- 4.66 p.p.m. and spw1 = 0.002. To achieve quantitative NMR, D, was set to 5 seconds and 90° pulse (p) to 11.69 µs.

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H2O stress experiments. The procedure was adapted from Christodoulou et al. with some modifications24. Cells were incubated in the experimental culture media, containing either [1,1-13C]ethanol or [1-13C]glucose, during the exponential growth stage. Metabolite extraction was performed with 1 ml of the culture for a baseline measurement (‘t’ time point). Afterwards, 6 µl of 30% hydrogen peroxide (Sigma-Aldrich) was added to the 3-ml culture to reach a final concentration of 20 mM H2O2 and samples were extracted after 15, 30 seconds and 60 seconds.

Mitochondrial acetyl-CoA labeling calculation from [U-13C]ethanol tracer. Acetyl-glutamate and glutamate labeling patterns were measured from the LC–MS analysis of cultures grown with [U-13C]ethanol. The matrix equation was set up as below and solved by the least square method for the acetyl-CoA labeling.

\[
\begin{align*}
M + 0 & \quad 0 \\
M + 1 & \quad M + 0 \\
M + 2 & \quad M + 1 \\
\end{align*}
\]

Gap Glutamate Acetyl-CoA Acetyl-glutamate

Water-soluble metabolite LC–MS analysis. Prepared samples were loaded onto a quadrupole-orbitrap mass spectrometer (Q Exactive Plus, Thermo Fisher Scientific) coupled to high-resolution liquid chromatography (HILIC) for analysis. Measurements of acetyl-CoA labeling were achieved by reversed-phase ion-pairing liquid chromatography coupled to a standalone orbitrap (Exactive, Thermo Fisher Scientific).

Fatty acid extraction and LC–MS analysis. Fatty acid extraction was performed according to Zhang et al. during the exponential growth stage. Metabolite extraction was performed with 1 ml of the culture for a baseline measurement (‘t’ time point). Afterwards, 6 µl of 30% hydrogen peroxide (Sigma-Aldrich) was added to the 3-ml culture to reach a final concentration of 20 mM H2O2 and samples were extracted after 15, 30 seconds and 60 seconds.
Data analysis and visualization. EL-MAVEN version 11.1 (Elucidata) software was used to process the LC–MS data. Metabolite identities were verified by both mass/charge (m/z) ratio and retention time match to authenticated standards. For 2H- and 13C-isotope-labeled data analysis, natural isotope abundance correction was made according to a binomial distribution model. 13C-MFA was computed with INCA. NADPH active hydride labeling and acetyl-CoA labeling from fatty acids were calculated as previously. Metabolite spectral accuracy on orbitraps. Anal. Chem. 89, 5940–5948 (2017).

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Author contributions
T.X. and J.D.R. designed the study. T.X. performed NMR studies. T.X. and A.K. performed isotopic tracing studies. T.X., Y.H., L.C. and A.K. developed the computational models. T.X. and A.K. analyzed the data. J.D.R. and T.X. wrote the manuscript, with the help from all authors.

Competing interests
The authors declare no competing interests.

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Extended Data Fig. 1 | Environmental ethanol consistently provides acetyl units across two S. cerevisiae strains. (a) Ethanol production rate from [U-13C]glucose (mean, SE, n=3 biological replicates). Results are similar to literature\(^\text{29}\) value of 10.5 mmol*(OD*h)\(^{-1}\). (b) Glucose uptake rate from the same experiment as in a (mean, SE, n=3 biological replicates), comparable to literature value\(^\text{29}\) of 6.9-7.6 mmol*(OD*h)\(^{-1}\). (c) The [M+2] labeling of acetyl-CoA in S. cerevisiae FY4 with equimolar glucose and [U-13C]ethanol measured as a function of time in labeled ethanol (mean, SE, n=3 biological replicates). The consistent labeling fraction from 30 min to 1 h implies that 1 h is a pseudo-steady-state measurement. (d) After incubation with glucose and [U-13C]ethanol for 1 hour at varying conditions and strains, the [M+2] labeled acetyl-CoA fraction from the cell (mixture of cytosolic and mitochondrial origins) was directly measured by LC-MS (mean, SE, n=3 biological replicates). (e) Examples of q\(^{13}\)C NMR spectra of the yeast culture media upon addition of [U-13C]glucose and after S. cerevisiae growth for 1 h.
Extended Data Fig. 2 | Environmental ethanol does not enter glycolytic intermediates in fermenting *S. cerevisiae*. (a) After natural isotope correction, no meaningful [M+2] fraction is observed in glycolytic intermediates: fructose-1,6-phosphate (FBP), dihydroxyacetone phosphate (DHAP), hexose-6-phosphates (G6P+F6P), or UDP-D-glucose (*S. cerevisiae*, equimolar glucose:[U-13C]ethanol, mean, SE, n=3 biological replicates). (b) As in a, for equicarbon condition. (c) Example of the natural abundance observed in raw mass spectra of FBP. The natural abundance is corrected by the binomial distribution model18 to arrive at the labeling patterns reported throughout the manuscript including a and b above.
Extended Data Fig. 3 | Environmental ethanol feeds fatty acid synthesis to a similar extent across the equimolar and equicarbon conditions.

(a) $^{13}$C-isotope labeling pattern of palmitic acid from *S. cerevisiae* FY4 (S288c) switched to and incubated in minimal media (YNB) with equimolar or equicarbon glucose: $[^{13}]C$ethanol (mean, SE, n=3 biological replicates). (b) $^{13}$C isotope labeling pattern of stearic acid from the same experiments as shown in a (mean, SE, n=3 biological replicates). (c) Whole-cell and cytosolic acetyl-CoA labeling from $[^{13}]C$ethanol is similar. Whole-cell labeling is directly measured by LC-MS of acetyl-CoA (mean, SE, n=3 biological replicates). Cytosolic labeling is inferred from fatty acids labeling patterns (mean ± SE, results from both C16:0 and C18:0 are averaged, resulting a total n=6 measurements from n=3 biological replicates). The directly measured whole-cell acetyl-CoA is the same data as shown in Fig. 1e.
Extended Data Fig. 4 | Ethanol contribution to mitochondrial acetyl-CoA is blocked by knocking out ACH1. (a) Glu and NAG labeling from the experiment in Fig. 1d with equicarbon glucose: [U-13C]ethanol (mean, SE, n=3 biological replicates; ***, p<0.001, by two-sided t-test). Briefly, while Glu is labeled up to [M+2], NAG [M+4] arises from the reaction of [M+2] Glu with mitochondrial [M+2] acetyl-CoA, which depends on Ach1. (b) Mitochondrial acetyl-CoA [M+2] fraction fitted from glutamate and NAG labeling in a (mean, SE, n=3 biological replicates). (c) Schematic showing ACH1 as the exclusive point of entry for carbons from ethanol or acetate into mitochondrial acetyl-CoA (Created with BioRender). (d) Directly measured cellular acetyl-CoA [M+2] labeled fraction is similar across media conditions or strains including the ACH1 knockout strain (mean, SE, RM one-way ANOVA with Geisser-Greenhouse correction, p=.097 (ns), 19(ns), n=3 biological replicates). FY4 is isogenic to S288c and Δach1 is from S288c, while CEN.PK is derived from ENYWA-1A and MC996A.
Extended Data Fig. 5 | Carbons from [U-13C]ethanol feed into TCA intermediates across media conditions and budding yeast strains/species. Some data for Sc. FY4 and I.o. SD108 are repeated from main Fig. 3b,c (mean, SE, n=3 biological replicates).
Extended Data Fig. 6 | The environmental ethanol contribution to TCA intermediates is concentration-dependent. Labeling pattern of TCA intermediates from the indicated budding yeast grown starting at OD = 0.1 in standard high glucose media (55 mM regular glucose) with the indicated concentrations of [U-13C]ethanol for 1 h (mean, SE, n=3 biological replicates). Note that, for the lower 13C-ethanol concentrations, the labeled ethanol is substantially diluted by unlabeled ethanol made from the unlabeled glucose during the duration of the experiment. In S. cerevisiae, this is about a 10-fold dilution for the 50 μM condition and 2-fold for the 500 μM condition. Thus, the above labeling patterns conservatively underestimate the contribution of low concentrations of environmental ethanol to TCA intermediates.
Extended Data Fig. 7 | Deuterium tracing into NAD(P)H is similar across media conditions and strains. (a) Scheme depicting NADP\(^+\)H and NADH production from \([1,1-2H_2]\)ethanol isotope tracer. (b) Labeled fractions of NADPH active hydride with the tracer in a. The values are computed from matrix decompositions of labeling distributions of NAD(P)H and NAD(P)\(^+\) that are directly measured by LC-MS (mean, SE, n=3 biological replicates). (c) As in b, for NADH (mean, SE, n=3 biological replicates). (d) Chemical scheme depicting NADPH\(^+\) production from \([1-2H]\)glucose via the first step of oxPPP. (e) Labeled fractions of NADPH active hydride with the tracer in d (mean, SE, n=3 biological replicates). (f) As in e, for NADH (mean, SE, n=3 biological replicates). (g) Chemical basis of D\(_2\)O active hydride exchange with NAD(P)H\(^+\). (h) The fraction of NADPH hydride exchanged with water, as measured in media swap experiment with 50% D\(_2\)O (mean, SE, n=3 biological replicates). (i) As in h, for NADH (mean, SE, n=3 biological replicates).
Extended Data Fig. 8 | ALD6 knockout shifts NADPH production (and thus fatty acid hydride labeling) towards the oxidative pentose phosphate pathway and ZWF knockout shifts it towards the ethanol-ALD6 pathway. (a) 2H isotope labeling pattern of palmitic and stearic acids from S. cerevisiae FY4 and Δald6 swapped into equimolar [1-2H]glucose: ethanol for 1 h (mean, SE, n=3 biological replicates). (b) As in a, for equicarbon [1-2H]glucose: ethanol (mean, SEM, n=3 biological replicates). (c) 2H isotope labeling pattern of palmitic and stearic acids from S. cerevisiae FY4 and Δald6 swapped into equimolar glucose: [1,1-2H2]ethanol for 1 h (mean, SE, n=3 biological replicates). (d) As in c, for equicarbon glucose: [1,1-2H2]ethanol (mean, SE, n=3 (biological replicates)).
Extended Data Fig. 9 | Fatty acids in S. cerevisiae are labeled from D₂O (50%) reflecting direct water incorporation during fatty acid synthesis and H-D exchange between water and NADPH. (a) ²H isotope labeling pattern of palmitic acid from S. cerevisiae FY4 swapped into regular glucose media with 50% D₂O for 1 h (mean, SE, n=3 biological replicates). (b) As in a, for stearic acid.
Extended Data Fig. 10 | The fructose-1,6-bisphosphate (FBP) pool size acutely increases in response to oxidative stress. FBP pool size, after treatment with 20 mM hydrogen peroxide in S. cerevisiae FY4 grown in glucose+ethanol. The increase in FBP pool size after the oxidative stress is statistically significant (mean, SE, n=6 biological replicates, slope > 0 by linear regression, p=0.03).
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Software and code

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Data collection
Thermo Fisher Scientific Xcalibur™ Software (4.0) is used for LC-MS data acquisition. Bruker TopSpin® Software (3.6.4) and ICON NMR (5) is used for NMR data acquisition. Accucor (R package) was used for natural isotope correction of tracer experiments.

Data analysis
MATLAB (R2018b), Python (3.6), R (3.6.3), El-MAVEN (0.10.0, 0.11.1), MestReNova (x64) and GraphPad Prism (9).

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| Sample size | Sample size for all experiments were ≥ 3, and were determined based on our prior experience on similar experiments and literature reports. No statistical methods were used to predetermine sample size. |
|-------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Data exclusions | No data was excluded from analysis. |
| Replication | Data are obtained from multiple biological replicates (n≥3) and all attempts are successful. |
| Randomization | Samples were randomized during LC-MS analysis (e.g. randomized loading order). Because the yeast used for analysis were from the same initial stock, no randomization on grouping was performed. |
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