Gene expression regulates metabolite homeostasis during the Crabtree effect: Implications for the adaptation and evolution of Metabolism

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A key issue in both molecular and evolutionary biology has been to define the roles of genes and phenotypes in the adaptation of organisms to environmental changes. The dominant view has been that an organism's metabolic adaptations are driven by gene expression and that gene mutations, independent of the starting phenotype, are responsible for the evolution of new metabolic phenotypes. We propose an alternate hypothesis, in which the phenotype and genotype together determine metabolic adaptation both in the lifetime of the organism and in the evolutionary selection of adaptive metabolic traits. We tested this hypothesis by flux-balance and metabolic-control analysis of the relative roles of the starting phenotype and gene expression in regulating the metabolic adaptations during the Crabtree effect in yeast, when they are switched from a low- to high-glucose environment. Critical for successful short-term adaptation was the ability of the glycogen/trehalose shunt to balance the glycolytic pathway. The role of later gene expression of new isoforms of glycolytic enzymes, rather than flux control, was to provide additional homeostatic mechanisms allowing an increase in the amount and efficiency of adenosine triphosphate and product formation while maintaining glycolytic balance. We further showed that homeostatic mechanisms, by allowing increased phenotypic plasticity, could have played an important role in guiding the evolution of the Crabtree effect. Although our findings are specific to Crabtree yeast, they are likely to be broadly found because of the well-recognized similarities in glucose metabolism across kingdoms and phyla from yeast to humans.

glucose metabolism | glycogen shunt | adaptation | homeostasis | Crabtree effect

For more than a 100 y, a central goal of biology has been to determine the roles that genes and phenotypes play in the adaptation of an organism to a change in the environment. In molecular biology, the dominant paradigm has been top-down genomic determination of phenotypes, including the pathogenesis of disease, a major motivation for the human genome project (1). However, this viewpoint has been challenged, both due to the increasing difficulty of mapping DNA elements to genes and functions (2) and failures to predict phenotypes from genotypes. McKnight (3), reviewing studies of cancer metabolism, proposed that, due to feedback, a strict top-down genomic control model was not sufficient and that direct studies of the metabolic phenotype were necessary to understand metabolic adaptation. In evolutionary biology, the Modern Synthesis of the mid-20th century was also a paradigm that gave genes a dominant role; it used changes in gene frequencies over many generations to explain evolutionary adaptations (4, 5). That view of allelic substitutions leading directly to the development of new phenotypes has been modified by the proposal that phenotypic plasticity, defined as the range of phenotypes that can be supported by a genotype (and its population variations), can extend the range of novel environments in which organisms can survive, allowing time for selection to supplement more rapid plastic responses with slower genetic changes (5, 6). In metabolism, the genetic changes involve gene expression, which takes minutes to hours; in evolutionary biology, the genetic changes involve mutations, changes in gene frequency, gene duplications, and the rewiring, via those mechanisms, of genetic control networks, all processes that take many generations. In both cases, plasticity precedes genetic change. These nuanced interactions are the subject of this paper.

The genome centric view of metabolic adaptation originated with Beadle (7), who introduced the concept, often summarized as "one gene, one enzyme," and used it to explain in born errors of metabolism. This concept was extended to coordinated expression of multiple genes in the pioneering work of Jacob and Monod (8), who showed in bacteria that the genes coding the enzymes in the lactose synthesis pathway are transcribed at an equal rate due to being on the same stretch of DNA that is under control of a common repressor element that binds RNA polymerase. In the absence of lactose, the polymerase is blocked by the repressor protein. When lactose in the environment increases, the repressor protein dissociates, allowing the polymerase to coordinately express the pathway enzymes, which results in a proportional increase in the lactose-consumption rate. They referred to this mechanism as an operon and described it as a "genetic regulatory mechanism" (8). These and subsequent findings led to the view that metabolic regulation and the

Significance

The dominant view in both molecular and evolutionary biology is that genotype controls the adaptation to new environments. We propose an alternate hypothesis, in which the phenotype and genotype both play important roles in metabolic adaptation in the lifetime of the organism and in the evolutionary selection of adaptive metabolic traits. When studying the Crabtree effect in yeast, we found that homeostatic mechanisms in the starting phenotype, primarily the glycogen/trehalose shunt, allow adaptation to high glucose. Subsequent gene expression, rather than creating a new phenotype, optimizes the initial adaptation by expression of new homeostatic mechanisms. Metabolic homeostatic mechanisms, by allowing increased phenotypic plasticity, could also have played an important role in guiding the evolution of the Crabtree phenotype.

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evolution of new metabolic phenotypes can be understood primarily from genomic analysis (1).

Though conceptually powerful, relatively simple operon mechanisms have not been found in eukaryotes (2). However, findings of coordinated gene expression, initially in yeast exposed to high glucose (9), have provided continuing—albeit indirect—support for top-down genomic regulation of metabolic adaptation. However, this conclusion has been challenged by studies using quantitative methods such as metabolic control analysis (10), which found that gene expression often does not have a major role in the adaptation of metabolic flux to environmental changes. Rather, the flux values after adaptation are often primarily determined by nongenomic mechanisms, such as allosteric activation and inhibition of enzymes (11, 12), and posttranslational enzyme modifications (13), such as phosphorylation (14, 15). Similar conclusions have been reached regarding mammalian glucose and mitochondrial metabolism (16–19). However, while in these systems, the primary adaptations in flux are driven by the initial metabolic phenotype, the accompanying widespread changes in gene expression remain largely unexplained.

To address the interplay of genomic and nongenomic mechanisms for metabolic adaptation, we reexamined the Crabtree effect (20, 21) in the yeast species *Saccharomyces cerevisiae*. The Crabtree effect (Fig. 1) describes the rapid shift of yeast energy metabolism from oxidation of nonglucose substrates (called the respiratory state) (Fig. 1*A*) to glycolytic consumption of glucose (early fermentation state) (Fig. 1*B*), when they are challenged with high glucose concentrations. Unlike an operon-type pathway, which cannot reach its maximum metabolic flux for many minutes due to the need to raise enzyme activities by gene expression, Crabtree yeast can reach their maximum rate of glucose metabolism soon after glucose exposure. It is one of many examples in unicellular organisms (22) and multicellular organisms (16–19) in which metabolic pathways can respond rapidly to a change in environment prior to a change in gene expression. Although specific to yeast, it is relevant to a wide range of organisms, including mammals, for it involves the coregulation of the ubiquitous glycolytic, gluconeogenic, and glycogen-synthesis pathways that play a central role in glucose metabolism.

When Crabtree yeast are first exposed to glucose, their glycolytic pathway is in an imbalanced state in which there are high activities of the enzymes that supply glucose to the glycolytic pathway, such as glucose transport (GT), hexokinase (HK), and phosphofructokinase (PFK), but reduced enzyme activity for the lower demand portion of glycolysis that couples the flux to cellular ATP needs, in particular, pyruvate kinase (PK) (Fig. 1*B*, "Early Fermentation State"). The initial relatively low enzyme activity of the demand portion of the pathway is important during the respiratory state, because it allows glucose 6-phosphate (G6P) production by gluconeogenesis for the pentose phosphate pathway (23). However, when there is a major influx of glucose into the cell, the resultant imbalance, if not compensated, leads to large increases of fructose bisphosphate (FBP) and G6P concentrations. The resultant collapse of ATP levels and cessation of glycolysis (*SI Appendix*, Fig. S4) can be lethal if the yeast are left in a high-glucose environment (24, 25). We and other groups have shown that this imbalance is compensated by the coordinate activation of the glycogen and trehalose synthesis and degradation pathways (26–29), which we have described as the glycogen/trehalose shunt (26, 30, 31). These pathways compensate for the flux imbalance by shunting G6P away from glycolysis to glycogen and trehalose synthesis (26), regulate inorganic phosphate (Pi) and ATP levels through futile cycling (28), and reduce the

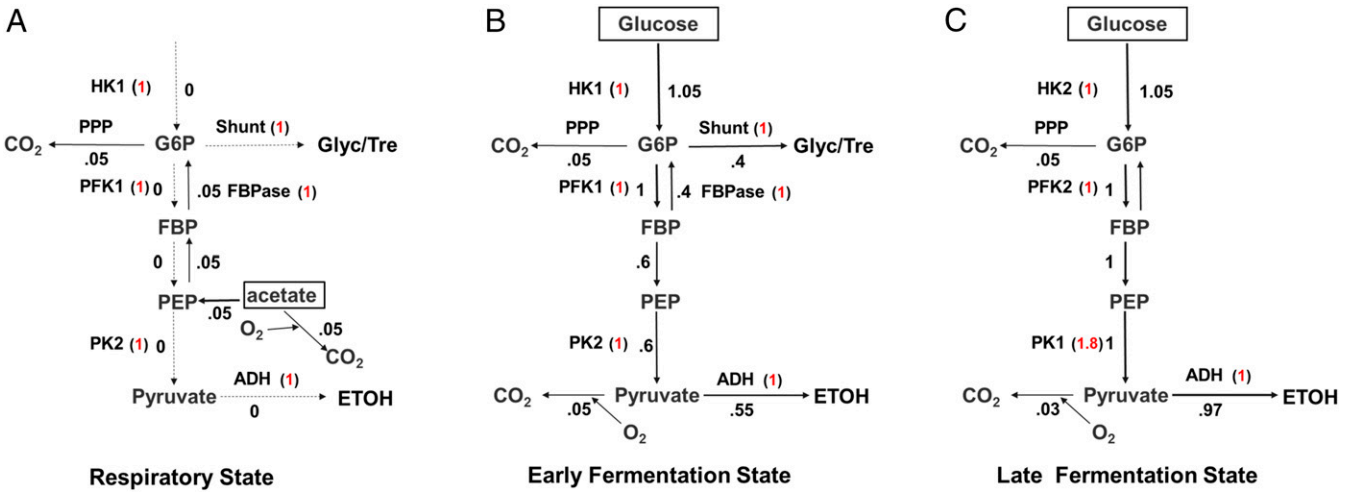

Fig. 1. Relative metabolic fluxes, enzyme activities, and dominant enzyme isoforms in the respiratory, early fermentation, and late fermentation states of the Crabtree effect. All fluxes are normalized to HK in the late fermentation state, which is set to a normalized flux of 1. All enzyme activities (in red) are normalized to their V_{max} value in the respiratory state. In all the studies analyzed to obtain metabolic fluxes, yeast were grown to the stationary state under glucose-restricted conditions (*Materials and Methods*) and then transferred to a high-glucose medium under aerobic conditions. Enzyme maximum activities and gene transcription rates were obtained from refs. 9 and 12 for yeast grown under similar conditions, as described in *Materials and Methods*. (*A*) Respiratory state. Oxidation of acetate (illustrated), ETOH, or other nonglucose substrates provides fuel for the tricarboxylic acid cycle. Gluconeogenesis through FBPase provides G6P for redox production in the pentose phosphate pathway. Fluxes were obtained using ^{13}C NMR (50, 51). (*B*) Early fermentation state upon addition of glucose. The system rapidly switches to fermentation with high flux through HK and PFK, reaching metabolic steady state in ~20 min, prior to changes in gene expression (9, 12). Fluxes and concentrations were obtained using ^{13}C and ^{31}P MRS under conditions (high-glucose nitrogen restriction) that, in stationary-state yeast, lead to rapid glycogen synthesis (50–54). Excess flux in the upper portion of glycolysis is converted to glycogen and trehalose via the glycogen/trehalose shunt. In addition, a futile cycle takes place between FBP and F6P/G6P due to continuing FBPase activity, which consumes ATP. The shunt and the FBPase futile cycle act to maintain metabolic homeostasis during the transition (Fig. 2). The remaining 60% of the glucose flux flows through the rest of the glycolytic pathway to be oxidized or form ETOH. (*C*) Late fermentation state. Due to reduced gene expression and posttranslational mechanisms, the activities of the glycogen/trehalose shunt and FBPase are repressed as well as glycogen and trehalose synthesis (9, 55). The flux through the upper portion of glycolysis remains the same (55), but the dominant isoforms of HK1 and PFK1 are replaced due to gene expression with the higher-affinity HK2 and PFK2 isoforms (9, 12, 32). PK similarly changes from predominantly the FBP-insensitive PK2 form to the highly sensitive PK1 isoform, which is associated with a 1.8-fold increase in its maximum activity (12), accompanied by a similar increase in the flux through the lower portion of glycolysis. Glyc/Tre, glycogen and trehalose; PPP, pentose phosphate pathway; shunt, glycogen shunt.

glucose influx by inhibition of HK (27). A diagram of these mechanisms, by which these pathways coordinate their flux with glycolysis to maintain homeostasis, is provided in Fig. 2.

Much less is known about the function of the large, stable changes in gene expression between early and late fermentation (Fig. 1*C*). During late fermentation, these changes in gene expression, as well as increased rates of enzyme breakdown, almost completely suppress the glycogen/trehalose shunt (9, 12, 13). However, there are no changes in the total activity of HK and PFK, or their flux, but instead the isoforms present in early fermentation, HK1 and PFK1 (which are expressed in the respiratory state), are replaced due to a shift in gene expression by new isoforms, HK2 and PFK2. These isoforms are more sensitive to allosteric regulation (9, 12, 32) (Fig. 2). Similarly, PK in the demand pathway, which is primarily the PK2 isoform in early fermentation, is largely replaced by PK1, which is much more sensitive to allosteric activation by FBP and phosphoenolpyruvate (PEP). Following these changes in gene expression, there is more ATP and ethanol (ETOH) produced per glucose consumed. However, there are no changes in the total glucose-consumption flux or the concentrations of the major glycolytic intermediates.

To explain the function of these changes in gene expression, we propose an alternative hypothesis: the role of gene expression in the late fermentation state is not primarily for controlling the glycolytic flux but rather to maintain homeostasis of glycolytic intermediates, thereby allowing increased ATP and ETOH production. To test this hypothesis, we applied a combination of flux-balance analysis (26, 28) and supply–demand analysis developed by Hofmeyr and coworkers (33, 34) to comprehensive published flux and glycolytic pathway intermediate-concentration data obtained during the early and late Crabtree effect.

Given the importance of the glycogen/trehalose shunt in allowing yeast to survive the switch to early fermentation, we also examined whether it could have played a role in the evolution of the Crabtree effect. We assumed that the ancestor of today's Crabtree yeast had low glycolytic activity during the respiratory state based on present non-Crabtree yeast-like organisms that share a common ancestry (35, 36). By simulation, we found that higher shunt activity, by providing phenotype plasticity, can

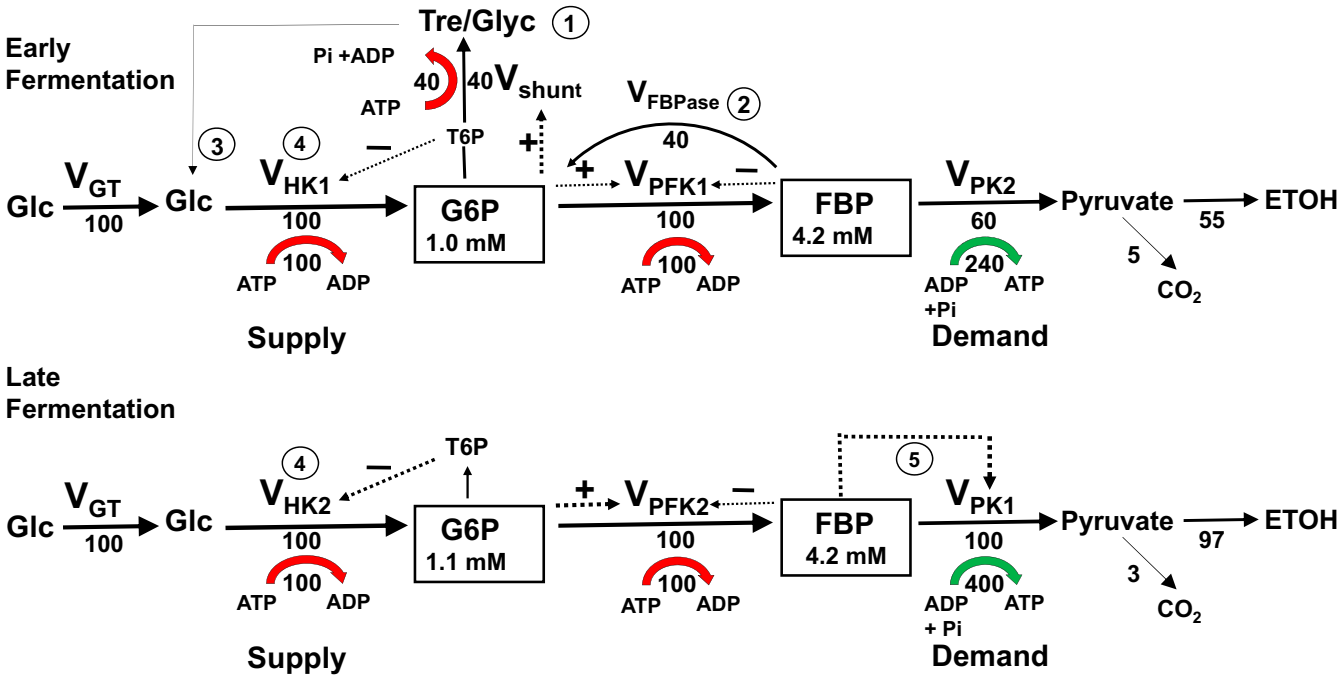

Fig. 2. Schematic of the supply–demand model of early and late fermentation and mechanisms for maintaining FBP, G6P, and ATP homeostasis during early (*Upper*) and late (*Lower*) fermentation under high-glucose aerobic conditions. The glycolytic pathway, the glycogen trehalose/shunt, and FBPase are grouped into a supply–demand pathway. For the supply portion of the pathway, the following enzymes are grouped together: **HK**, GT and HK; **PFK**, phosphoglucose isomerase, PFK, and PFK-2/FBPase 2, which regulates the level of the allosteric effector F2,6BP (see *SI Appendix*, section 3 for a detailed description); and **FBPase**. The demand pathway, **PK**, consists of all of the glycolytic enzymes after FBP. Allosteric regulation of each group of enzymes is indicated by dashed lines and arrows. Velocities of each enzyme group are represented by V_{HK}, V_{PFK}, V_{shunt}, and V_{PK} and are normalized to the velocity through HK in the early fermentation state (V_{HK1}), which is set to 100. (*Upper*) Early fermentation state (glycogen/trehalose synthesis). Under nitrogen starvation conditions, G6P and FBP concentrations are regulated by storage of the excess flow from glucose into G6P through glycogen synthesis ($\mathbf{V_{shunt}}$, **mechanism 1**). ATP consumption due to glycogen synthesis and futile cycling between PFK and FBPase balances ATP synthesis by the demand pathway ($\mathbf{V_{FBPase}}$, **mechanism 2**). Based on flux-balance analysis, the relative fluxes shown are required to maintain steady-state levels of G6P, FBP, and ATP, in good agreement with experimental measurements (26, 28). (*Upper*) Early fermentation state (no glycogen synthesis/trehalose futile cycling). Under conditions when nitrogen sources are present during early fermentation, yeast do not synthesize glycogen and trehalose. The glycolytic flux imbalance is compensated for by inhibition of HK velocity due to an increase in the concentration of the intermediate in the trehalose shunt pathway T6P (**mechanism 3**) (27). ATP homeostasis is maintained through trehalose and futile cycling (**mechanism 4**) (28), which also helps regulate Pi levels allowing the velocity of PK to increase to match the supply velocity. Once these velocities reach steady state, trehalose futile cycling declines. (*Lower*) Late fermentation state. Changes in gene expression and increased posttranslational enzyme degradation results in suppression of the glycogen/trehalose shunt and FBPase activity. The concentrations of G6P and FBP, and the fluxes through HK and PFK, remain constant, but there is a fivefold drop in the concentrations of the allosteric modulators T6P and F2,6BP (12). The predominant glycolytic enzyme isoforms due to gene expression become HK2, PFK2, and PK1 (9, 12, 23, 32). These changes result in enhanced allosteric sensitivity for activation of PK by FBP (**mechanism 5**) and, due to higher T6P and F2,6BP affinity, compensate for the drop in their concentration maintaining the elasticity of HK at early fermentation levels (**mechanism 4**) while increasing the elasticity of PFK to F6P (**mechanism 6**). Additional homeostatic regulation, by balancing the supply and demand relative glycolytic intermediate levels, is provided by F2,6BP (**mechanism 7**).

increase the percentage of a yeast population with enhanced glycolytic activity that can survive a glucose challenge. As a consequence, if the environment changes so that increased glycolytic activity provides a survival advantage, the population has enough high-glycolytic phenotypic variants so that it can survive the many generations needed for the evolution of the present Crabtree phenotype.

Overall, we conclude that, for the short-term adaptation over the lifetime of an organism and the long-term selection of adaptations over many generations, the metabolic phenotype plays a key role in early adaptation and in guiding subsequent short-term changes in gene expression and long-term genomic adaptations. Although our findings are specific to the Crabtree effect, they likely hold broadly because of the well-recognized similarities in glucose metabolism between different pathways and across kingdoms and phyla from yeast to humans.

Results

Supply/Demand Model for Calculating FBP and G6P Concentration Control Coefficients for Changes in Supply-Pathway Enzyme Activity. Previously, we (26) and van Heerden et al. (28) performed flux-balance analysis for the early fermentation state and showed how coordination between the fluxes through the glycogen/trehalose shunt compensated for the initial imbalance in glycolysis during early fermentation. To assess whether gene expression during late fermentation has a similar homeostatic function, we analyzed the pathway using a supply–demand model (33, 34), in order to calculate concentration and flux control coefficients. These coefficients provide quantitative measures of the control that specific enzymes or groups of enzymes exert on the flux of a pathway and the concentration of pathway intermediates. The advantages of using a supply–demand pathway analysis are that it provides a conceptual framework for understanding how pathway and metabolic network structures interact with enzyme kinetic properties to regulate fluxes and concentrations, while providing theoretical tools for determining flux and concentration control coefficients even when complete kinetic descriptions of the enzymes and regulatory mechanisms in the system are not available (12, 33, 34, 37).

A schematic of the model, and the main allosteric regulatory mechanisms incorporated in it, is shown in Fig. 2. The supply portion of the pathway consists of GT, all of the glycolytic enzymes above FBP, fructose bisphosphatase (FBPase), and the enzymes in the glycogen/trehalose shunt. The demand pathway consists of all of the enzymes below FBP up until the final product, pyruvate/lactate. To simplify the description, we grouped adjacent enzymes within the pathways and refer to each group by the name of the enzyme believed to primarily determine the group kinetic properties (HK, PFK, FBPase, shunt, PK), as described in *SI Appendix*, section 1. The internal allosteric effectors included in the supply pathway are F6P, G6P, and T6P. FBP is the shared intermediate between the supply and demand pathways. Also included in the description is the level of fructose 2,6-bisphosphate (F2,6BP), which is one of the primary allosteric activators of PFK. It is assumed in that when there are changes in the activity of the supply or demand pathway enzymes, which are required for the analysis, that allosteric effectors from outside the system other than F2,6BP (for example ATP, ADP) do not change their concentrations and there are no changes in enzyme phosphorylation state (or other posttranslational modifications).

For the mathematical description of the model, we wrote a total fractional differential of the velocity (V) of each enzyme group in the model with respect to a fractional change in its total activity (dE/E) and fractional changes in the concentration of its substrates, products, and allosteric effects. Eq. **1** shows an example of this linear expansion for the fractional velocity of PFK in the supply portion of the pathway:

$$dV_{PFK}/V_{PFKo} = (dE_{PFKt}/E_{PFKo}) + \varepsilon^{VPFK}_{F6P} * (dF6P/F6P) + \varepsilon^{VPFK}_{F2,6P} * (dF2,6P/F2,6P) + \varepsilon^{VPFK}_{FBP} * (dFBP/FBP). \quad [1]$$

The term E_{PFKo} is proportional to the maximum activity of the enzymes in the PFK group. The terms ε^{VPFK}_{F6P}, $\varepsilon^{VPFK}_{F2,6P}$, and ε^{VPFK}_{FBP} are elasticity coefficients that give the fractional change in velocity of PFK with respect to a fractional change in F6P, FBP, and F2,6BP concentration. In addition, flux-balance equations were written for the G6P and FBP pools. Two additional mass balance equations were included about the FBP and combined F6P/G6P pool. The equations were further simplified by expressing both F6P and T6P in terms of G6P. Because it is not known whether F2,6BP concentrations are affected by changes in glycolytic intermediates under fermentation conditions, we performed our calculations with dF2,6BP/F2,6BP = 0 and then assessed analytically the impact of including the F2,6BP synthesis system in the model (*SI Appendix*, section 3).

The full system of equations was then solved to simulate the effect of an increase in the fractional activity of all enzymes in the supply pathway by $dE_{supply}/E_{supplyo}$. For the example of Eq. **1**, $dE_{PFKt}/E_{PFKo} = dE_{supply}/E_{supplyo}$. The concentration control coefficients were then calculated according to refs. 33 and 34 using the following formulae:

$$dFBP/FBP = C^{FBP}_{supply} * (dE_{Supply}/E_{Supplyo}), \quad [2A]$$

$$dG6P/G6P = C^{G6P}_{supply} * (dE_{Supply}/E_{Supplyo}). \quad [2B]$$

Eq. **2A** describes how the concentration control coefficient relates a fractional change in the supply-pathway enzyme activity to a fractional change in FBP concentration. A similar equation for G6P is shown in Eq. **2B**. Because G6P is internal in the supply portion of the pathway, Eq. **2B** is the combined concentration control coefficient for G6P of each of the supply enzymes. As seen in these equations, the concentrations of FBP and G6P become more stable to changes in supply enzyme activity as the flux control coefficients approach 0 (33, 34).

The choice of calculating control coefficients for the combined supply-pathway activity, as opposed to individual enzymes in it, is based on the finding that their activities increase and decrease in parallel, as do the demand-pathway enzymes (9, 12). However, similar results were found by selectively increasing the fractional activity of just GT and HK, which simulates the effect of an increase in glucose concentration.

The results of the calculations are given in the legend of Fig. 3 and described in the sections below. The determination of in vivo elasticity values is described in detail in *SI Appendix*, section 3. Because, in several cases, elasticities were determined from isolated enzymes, which may not accurately reflect the behavior of a group of enzymes in vivo (12, 33, 34), we performed sensitivity analysis in which we varied each elasticity from 0- to 2-fold greater than the measured values, which is well beyond the anticipated measurement variation. We also used these simulations, presented in more detail in *SI Appendix*, sections 1 and 3, to assess the contributions of individual enzymes/enzyme group activities and elasticities to the concentration control coefficients and therefore maintenance of FBP and G6P homeostasis.

During Early Fermentation Regulation of G6P and FBP, Homeostasis Depends on the Coordinate Regulation of Glycolysis with the Glycogen/Trehalose Shunt. We calculated that due to the high elasticity of the shunt to G6P (16–18), there was an ~10-fold decrease in the concentration control coefficients of FBP and G6P to changes in the supply pathway relative to the case where

shunt activity (or elasticity) was set to 0 along with the related elasticity of HK to T6P (Fig. 3*A*, blue versus orange bars). The coordinated increase in the demand for G6P and ATP by the shunt and futile cycling is a specific mechanism for achieving the general requirement of supply/demand pathways that an increase in supply flux needs to be balanced by a matching increase in demand flux to maintain concentration homeostasis (37). The concentration control coefficients were also robust to the elasticity and total activity of the shunt, with a fivefold reduction from the in vivo value needed before there was a significant change in the supply concentration control coefficients (Table 1 and *SI Appendix*, Fig. S1).

Under conditions of no net glycogen/trehalose synthesis, the elasticity of HK to T6P was sufficiently high that the supply concentration control coefficients were only approximately twofold higher than if there was net glycogen/trehalose synthesis (*SI Appendix*, section 4). This finding is consistent with previous reports that this mechanism (Fig. 2), in combination with trehalose futile cycling, allows for G6P and FBP concentrations to reach a stable steady state (28) during early fermentation. Varying the elasticities of PFK and FBPase to F6P and FBP from 0 to 2 times their in vivo values had only minor effects on the supply concentration control coefficients.

During Late Fermentation Expression of the HK2, PFK2, and PK1 Isoforms Is the Primary Mechanism for Maintaining FBP and G6P Homeostasis. During late fermentation, the activity of the shunt and FBPase are suppressed by reduced gene expression (9) and posttranslational mechanisms (11). A similar suppression of the HK1, PFK1, and PK2 isoforms takes place. Increased gene expression of PK1 isoforms lead to an approximate 25-fold increase in its elasticity to FBP (23, 32) as well as a 1.8-fold higher maximum activity (12). While concentrations of G6P, F6P, and FBP remain approximately the same, the concentrations of T6P

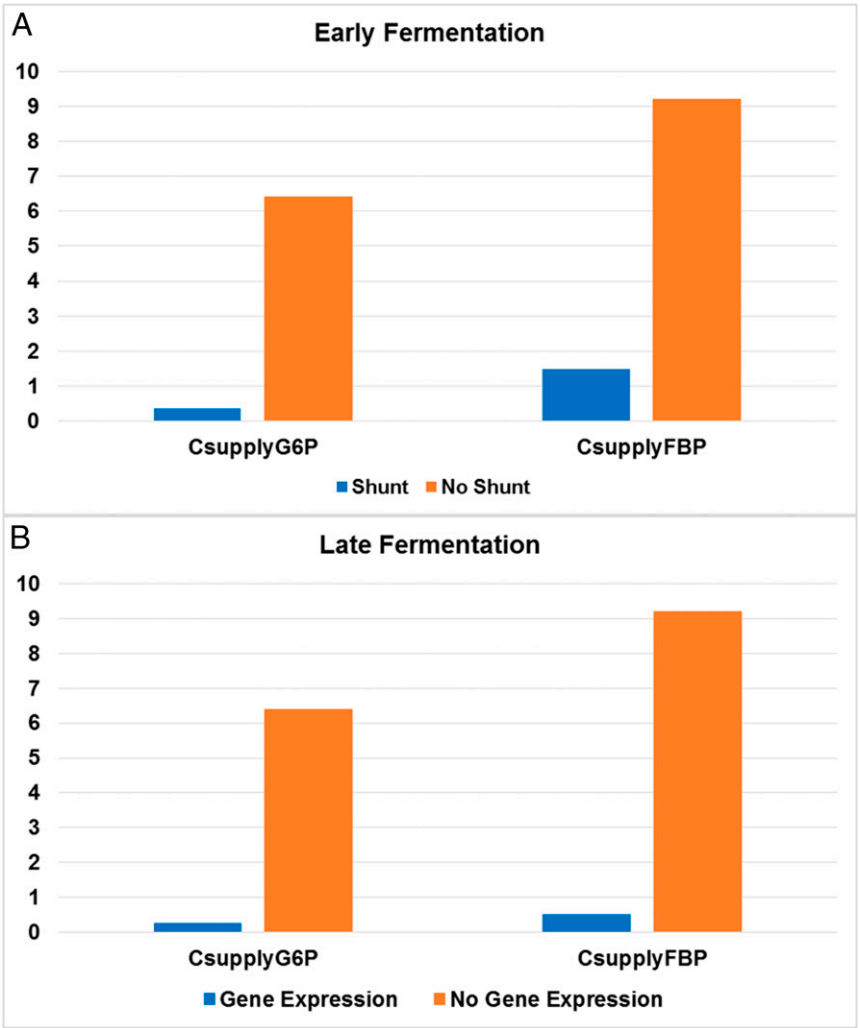

Fig. 3. (*A*) Effect of the glycogen/trehalose shunt on the concentration control coefficients of FBP and G6P to changes in supply-pathway enzyme activity during early fermentation The bar graph shows the calculated supply concentration control coefficients for G6P and FBP (C_{supply}^{G6P}, C_{supply}^{FBP}). When the shunt is at its in vivo activity, the supply concentration control coefficients are $C_{supply}^{G6P} = 0.36$ and $C_{supply}^{FBP} = 1.48$ (blue). In the absence of the shunt homeostatic mechanisms (orange), the imbalance in the activity of the supply and demand portions of the pathway is not compensated for, resulting in very high concentration control coefficients of $C_{supply}^{G6P} = 6.4$ and $C_{supply}^{FBP} = 0.2$, consistent with the loss of G6P and FBP homeostasis found in mutants that block shunt activity (24, 25). (*B*) Effect of gene expression of HK2, PFK2, and PK1 on the concentration control coefficients of FBP and G6P to changes in supply-pathway enzyme activity during late fermentation. The bar graph shows the calculated supply concentration control coefficients for G6P and FBP during late fermentation. The replacement of the isoforms during early fermentation with isoforms with higher binding affinity and elasticity values (HK2, PFK2, PK1) results in low supply concentration control coefficients ($C_{supply}^{G6P} = 0.17$ and $C_{supply}^{FBP} = 0.46$) (blue). If this isoform replacement did not occur, the supply concentration control coefficients would be the same as in early fermentation if the shunt mechanisms were blocked ($C_{supply}^{G6P} = 6.4$ and $C_{supply}^{FBP} = 9.2$).

Table 1. Concentration control coefficients, flux control coefficients, elasticities, and relative fluxes (to $V_{supply\ glycolysis} = V_{HK}$) during early and late fermentation

	Early fermentation	Late fermentation
Concentration control coefficients		
C_{supply}^{FBP}	1.48	0.36
C_{supply}^{G6P}	0.36	0.17
Flux control coefficients		
$C^{Vdemand}_{demand}$	~1	~0
$C^{Vdemand}_{supply}$	0	~1
Elasticities		
ε^{VHK}_{T6P}	0.43	0.43
$\varepsilon^{Vshunt}_{G6P}$	2.5	0.0
ε^{VPFK}_{F6P}	0.75	1.0
ε^{VPFK}_{FBP}	0.53	0.53
$\varepsilon^{VPFK}_{F2,6BP}$	1.0	1.0
$\varepsilon^{VFBPase}_{FBP}$	0.0	0.0
$\varepsilon^{VFBPase}_{F6P}$	0.0	0.0
ε^{VPK}_{FBP}	0.10	2.0
Relative fluxes (to $V_{supply\ glycolysis}$)		
$V_{supply\ glycolysis}$	1	1.0
$V_{demand\ glycolysis}$	0.6	1.0
V_{shunt}	0.4	0.0
V_{FBPase}	0.4	0.0
V_{ETOH}	0.55	0.97
$V_{ATP}/V_{supply\ glycolysis}$	0	2.0

The fluxes apply to conditions in which yeast are grown in the respiratory state until they reach the stationary state and are then exposed to high glucose in a starvation medium (no nitrogen sources). When a nitrogen source is present, there is no glycogen/trehalose synthesis, and the activity of HK is inhibited by T6P to balance the supply and demand fluxes, which reduces $V_{supply\ glycolysis}$ to 0.6 in early fermentation. When glycerol synthesis takes place, the flux through the demand glycolysis pathway will be reduced as will ETOH synthesis.

and F2,6BP drop by approximately fivefold (12). However, due to the higher affinity of HK2 to T6P (38), its elasticity to T6P does not change relative to early fermentation (*SI Appendix*, section 3). Similarly, due to the higher affinity of PFK2 to F2,6BP binding, and other changes, its elasticity to F6P and FBP increases relative to PFK1 in early fermentation despite the lower F2,6BP concentration (*SI Appendix*, section 3).

To determine whether these changes provide additional homeostatic mechanisms, we calculated the concentration control coefficients for G6P and FBP in response to a change in supply enzyme activity using experimentally determined values for HK, PFK, and PK elasticities in the late fermentation state (Table 1 and *SI Appendix*, section 3). As shown in Fig. 3*B*, the values of the concentration control coefficients with respect to changes in supply-pathway activity were approximately twofold lower in late fermentation than in early fermentation, demonstrating that these mechanisms provide effective concentration regulation. In contrast, if these isoform changes did not occur, the late-fermentation supply concentration control coefficients would increase to the same very high values as in early fermentation in the absence of the shunt-related mechanisms (Fig. 3, orange bars).

By varying the elasticity values of HK, PFK, and PK during late fermentation from 0 to 2 times their in vivo values, we found that the high elasticity of PK1 to FBP, ~2.0, was the primary homeostatic mechanism (23, 32) (*SI Appendix*, Fig. S2). The PK elasticity value in late fermentation is ~25 times higher than during early fermentation (29) (Table 1). The supply-pathway concentration control coefficients were robust to the elasticity of PK to FBP. An almost 5× reduction in the elasticity was required to raise the concentration control coefficients above 1.0 (*SI Appendix*, Fig. S2). The key role of PK1 in maintaining FBP homeostasis can also be understood from theoretical considerations of supply–demand control theory (37). For a linear supply–demand pathway, the supply and demand concentration control coefficients to the intermediate is given as, for FBP:

$$C_{supply}^{FBP} = 1 \Big/ \left[\varepsilon_{FBP}^{Vsupply} + \varepsilon_{FBP}^{Vdemand} \right], \qquad [3]$$

where the elasticities are absolute values. As seen from Eq. **3**, the high value of $\varepsilon^{Vdemand}_{FBP}$ due to expression of PK1 by itself is sufficient to maintain a low value of C_{supply}^{FBP}.

We assessed the effect of the elasticity of PFK to F6P and FBP during late fermentation on the supply-pathway concentration control coefficients by performing simulations in which we varied the elasticities from 0 to 2 times their in vivo values. Based on these simulations (*SI Appendix*, Fig. S3), the supply concentration control coefficient for FBP was insensitive to PFK elasticity, as predicted by Eq. **3**. In contrast, the supply concentration control coefficient for G6P was sensitive to the elasticities of PFK to F6P and FBP in a reciprocal manner, decreasing with higher elasticity to F6P and increasing with higher elasticity to FBP. The increased elasticity of HK2 to T6P, as a consequence of its increased affinity relative to HK1 (38), also had minimal impact on C_{supply}^{FBP} (*SI Appendix*, Fig. S3). However, it potentially provides a safeguard under situations where PK1 activity undergoes a large degree of inhibition by preventing a large increase in the supply concentration control coefficients, as it does in early fermentation.

We also examined the impact of the elasticity of PFK to F2,6BP on the calculated supply concentration control coefficients. F2,6BP is considered to be a major allosteric effector of PFK, with its level in yeast being increased by cyclic adenosine monophosphate (cAMP)-dependent phosphorylation of its synthesis enzyme 6-phosphofructo-2-kinase (6PFK-2) (39). Under in vivo conditions, its velocity increases with F6P concentration and is inhibited by FBP, glycerol 3 phosphate (G3P), and PEP. The enzyme for its

breakdown, fructose-2,6-bisphosphatase (F2,6BPase), is present at a much lower total activity and is inactivated by cAMP-dependent phosphorylation as well as by inhibition by F6P (39). As shown in Eq. **1**, its effect on the fractional velocity change of PFK depends on both the elasticity coefficient to F2,6BP and the fractional change in F2,6BP concentration.

Based on the severalfold decrease in F2,6BP concentration (12) in going from early to late fermentation, it is likely that PFK2 has a higher affinity to F2,6BP than PFK1. In order to assess the role of this higher affinity in maintaining concentration homeostasis, we modeled the F2,6BP synthesis/breakdown system together with the glycolytic pathway. We derived the following equation that describes the relationship between the concentrations of F6P, FBP/PEP, and F2,6BP (*SI Appendix*, section 3):

$$dF6P/F6P = \left(\varepsilon^{*V6PFK\text{-}2}_{FBP} / \varepsilon^{*V6PFK\text{-}2}_{F6P}\right) * (dFBP/FBP), \qquad [4]$$

where $\varepsilon^{V6PFK\text{-}2}{}_{F6P}$ is the elasticity of 6PFK-2 to F6P in the supply pathway, and $\varepsilon^{*V6PFK\text{-}2}{}_{F6P}$ is the combined elasticity of 6PFK-2 to FBP and PEP in the demand pathway.

Eq. **4** shows that 6PFK-2, through F2,6BP, acts to balance the concentrations of F6P in the supply pathway and PEP in the demand pathway, with the shared intermediate FBP. A test of this proposed role is provided by studies in which the sensitivity of PFK2 to F2,6BP was eliminated (40, 41). There was surprisingly no impact on the flux through PFK. However, the authors found, consistent with Eq. **4**, that the ratio of F6P/FBP increased from 0.5 in wild type yeast to 2.0 in the engineered yeast. Additional insight into this homeostatic role for F2,6BP may be obtained through more comprehensive enzyme kinetic modeling of the combined glycolysis and F2,6BP synthesis system (29).

Gylcolytic Isoform Changes during Late Fermentation Increase ATP and ETOH Production by Allowing Suppression of Glycogen/Trehalose Synthesis and Futile Cycling without a Loss of Glycolytic Intermediate Homeostasis. Previous studies have shown that under this condition, flux control of glycolysis, including the demand pathway that synthesizes ATP, is shifted to the GT/HK steps in the supply pathway (11, 12, 42–44). From our analysis, this shift in flux control is an additional consequence of the much higher elasticity, and 1.8 times higher activity, of the PK1 isoform expressed in late fermentation. By shifting flux control to steps in the supply pathway, and playing a major role in maintaining low supply concentration control coefficients, the isoform changes due to gene expression both allow high demand flux and result in higher ATP and ETOH production (Fig. 2 and Table 1).

Homeostatic Regulation by the Glycogen/Trehalose Shunt Can Enhance Glycolytic Phenotype Plasticity. We addressed whether the glycogen/trehalose shunt could have led to increased phenotypic plasticity of glycolysis in the ancestors of present Crabtree effect yeast. Our approach was to perform simulations of whether the presence of the shunt allowed a higher percentage of yeast with elevated supply pathway to survive an environmental glucose challenge. Fig. 4*A* shows curves of FBP level (in micromoles per gram dry weight) versus the fractional increase in supply-pathway enzyme activity relative to wild type (normalized to 1 for comparison) during early exposure to high-glucose conditions. The wild-type activities of HK, PFK, and PK were assumed to be low and balanced and their kinetic properties similar to present Crabtree yeast in the late fermentation state (primarily HK2, PFK2, PK1), as is believed to be the case for the common ancestor of Crabtree and non-Crabtree yeast species (35, 36, 45). The low elasticity isoforms such as HK1, PK1, and PK2 are believed to have evolved after the lineage of present Crabtree yeasts diverged (45).

The relative activity of the shunt to the glycolytic pathway was expressed in terms of the concentration control coefficient for FBP with respect to a change in the supply pathway, with a value of 10 when there is no shunt activity to a minimum value of 0.5 for high shunt activity. As shown in the plot, at low values of shunt activity (high concentration control coefficients) the elevation in FBP concentration with increasing supply enzyme activity can reach potentially lethal levels (Fig. 4*A*) even for small fractional increases in supply glycolytic enzyme activity.

To assess the hypothetical impact of the shunt on the ability of yeast to survive a glucose challenge, we performed a simulation in which the probability of yeast surviving a single high-glucose exposure was dependent on FBP levels. We assumed a normal survival distribution with 50% lethality when FBP concentration reached twice the median level in the population, based upon the level of FBP reached that was sufficient to cease glycolysis in the cif mutation (24, 25). Fig. 5*B* shows the calculated survival curves as a function of the fractional increase in supply glycolytic enzyme activity. When the shunt has a relatively high activity (FBP concentration control coefficients for changes in supply enzyme activity on the order of 1.0 and below), FBP concentrations are well regulated and there is close to 100% survival. In contrast, when there is low shunt activity (high FBP concentration control coefficients to a change in supply enzyme activity), only yeast with small to no increases in supply enzyme activity have a high survival percentage.

Fig. 5 uses the same survival distribution as a function of FBP concentration as Fig. 4*B*, but it assumes the yeast population has a normal distribution of supply enzyme activity relative to the median of the population. The positive half of the distribution is shown. When shunt activity is relatively low, even a single exposure to high glucose is sufficient to narrow the variation in glycolytic supply enzyme activity in the population. Further narrowing of the distribution occurs with multiple exposures to high glucose, with the example of five exposures shown. However, yeast with a high shunt activity, which stabilizes FBP levels, are able to survive.

Although the curves in Fig. 5 are hypothetical, the predicted positive relation between shunt activity and survival of imbalanced glycolytic phenotypes is supported by recent results from van Heerden et al. (28). Using single-cell methods, they found that, in a Crabtree yeast population, not all of the yeast were able to activate glycolysis during high-glucose exposure. Based on studies of the cif and other mutations, it is likely that if kept in the medium, they would have died or failed to grow (24, 25). The yeast that failed to adapt had decreased activity of shunt-related homeostatic mechanisms (mechanisms 3 and 4 in Fig. 2) due to random variations in gene expression. The percentage of yeast that failed to adapt increased with decreases in shunt activity, as predicted in our simulations.

Discussion

The primary function of gene expression in the Crabtree effect is not to adapt the cell to glucose metabolism, which is largely accomplished by posttranslational mechanisms inherent in the respiratory state. Instead it fine tunes the adaptation, resulting in increased glycolytic ATP and ETOH production (Table 1). This fine tuning is performed through expression of enzyme isoforms with enhanced allosteric sensitivity to G6P and FBP concentrations and suppression of ATP consumption by the shunt and FBPase futile cycling (Fig. 2). The enhanced allosteric sensitivity maintains homeostasis of FBP and G6P concentrations. We address below general implications of these findings for the adaptation of metabolism to changes in the environment during the life of an organism and over many generations through evolutionary adaptation.

Given the higher ATP and ETOH production during late fermentation, the question arises, why do yeast not just maintain the late fermentation glycolytic pathway always? A potential

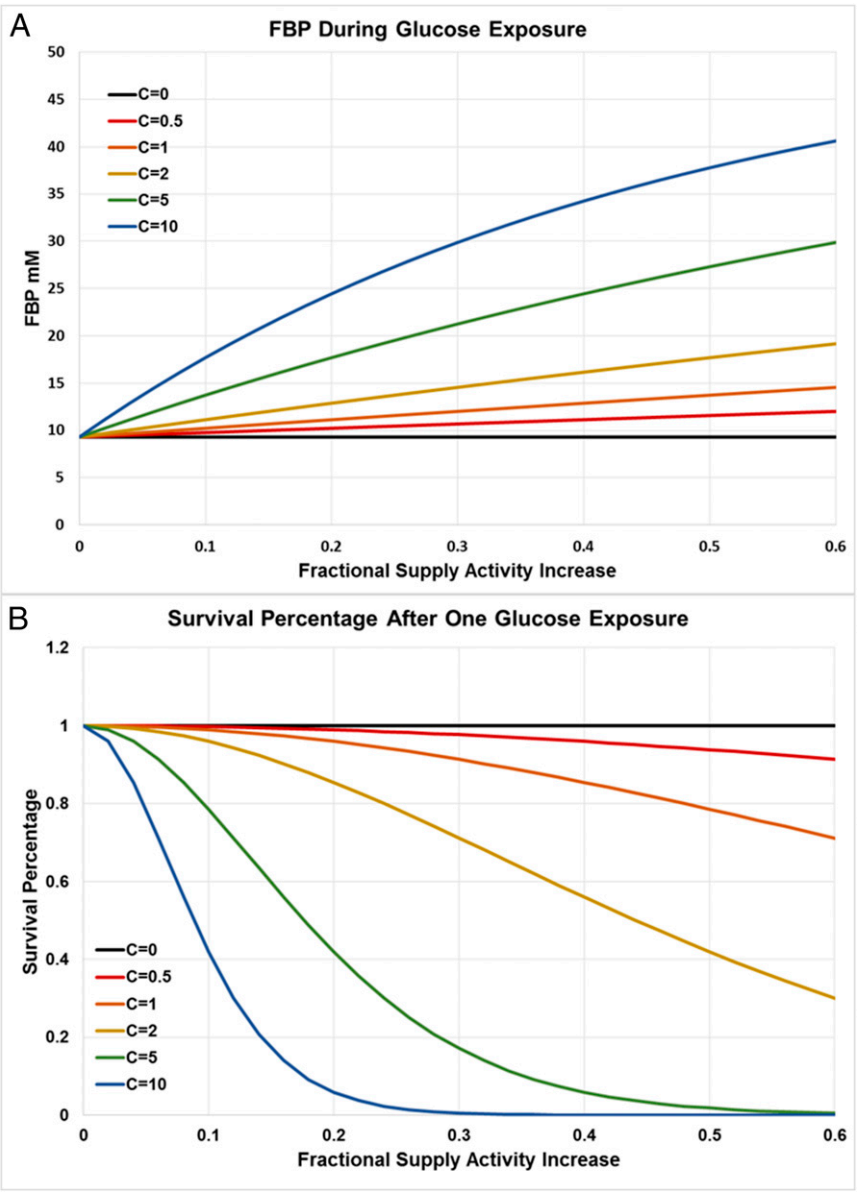

Fig. 4. Simulated curves of FBP levels and percentage survival versus glycolytic supply activity during exposure to high glucose. Calculations are described in *Results* and *SI Appendix*. "C" in the graph refers to the supply concentration control coefficient for FBP (C^{FBP}_{supply}). (*A*) Calculated FBP levels, in micromoles per gram dry weight, during early fermentation are shown as a function of an increase in the fractional activity of the enzymes in the supply portion of glycolysis (dEs_{supply}/E_{supply}). Curves are calculated ranging from maximum possible shunt activity ($C^{FBP}_{supply} = 0$) to no shunt activity ($C^{FBP}_{supply} = 10$). The elasticity of the shunt is maintained constant. (*B*) Survival percentage for yeast shown as a function of the fractional increase in supply glycolytic activity after one exposure to high glucose. The curves are for different levels of shunt activity, with 100% survival when $C^{FBP}_{supply} = 0$.

explanation is that in the respiratory state, the yeast need gluconeogenesis to produce G6P for the pentose shunt (Fig. 1). High PK activity and activation by FBP has been proposed to potentially short circuit this process (23, 32, 45), resulting in a wasteful futile cycle between PEP and pyruvate. Direct evidence for this possibility is found in a recent study on fission yeast, which only express a PK isoform with a low affinity for FBP, similar to PK2. The yeast showed a large reduction in O_2 consumption during the respiratory state when made to express instead the high activity FBP/PEP sensitive PyK1p form (46). Furthermore, studies looking at the transition from late fermentation to respiration found that the PK1 isoform immediately undergoes near-complete inhibition and then rapid degradation by posttranslational mechanisms (32) and concluded that its high sensitivity to allosteric activation by FBP and PEP makes the PK1 isoform incompatible with the requirements of the respiratory state for gluconeogenesis.

Recently a challenge to this explanation was presented by Solis-Escalante et al. (45), who engineered yeast to delete transcription of the majority of the minor isoforms of glycolytic enzymes. These minor isoforms correspond to the ones expressed during early fermentation, including HK1 and PK2. They found that these engineered yeast, referred to as minimal glycolysis yeast, had similar growth rates, and glycolytic intermediate concentrations, as wild-type *S. cerevisiae* under both glucose restricted (ETOH as carbon source) and high-glucose conditions. In addition, the rates of transcription of the major isoforms was the same as wild type in all conditions studied, suggesting similar enzyme activities. We feel this paradox can be resolved based on our findings of the relative roles of these isoforms in the different

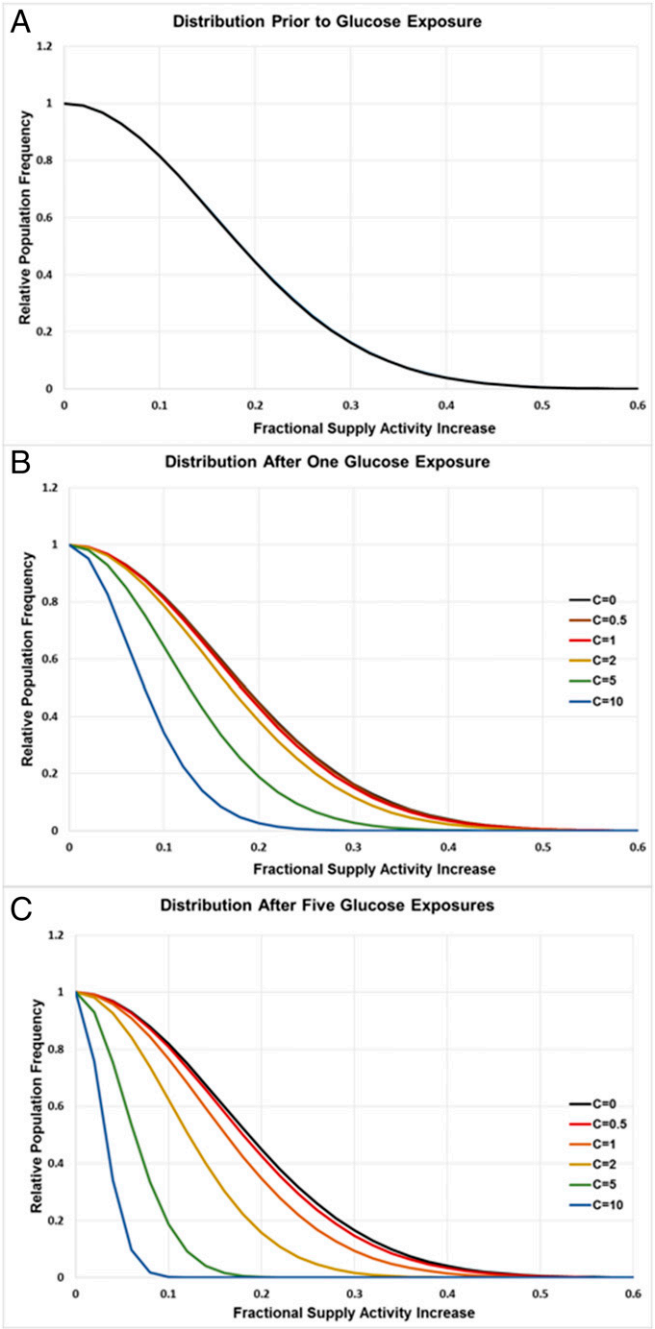

Fig. 5. Simulated effect of repeated glucose exposures on the glycolytic phenotypic plasticity of yeast as a function of shunt activity. See *Results* and *SI Appendix* for details of the calculations. "C" in the graph refers to the supply concentration control coefficient for FBP (C^{FBP}_{supply}). (*A*) The assumed normal population distribution of yeast as a function of the fractional increase from the median value of the supply HK/PFK activity in the glycolytic pathway. Only the right side of the curve is shown. The *y* axis gives relative frequency in the population as a function of the increase in the fractional activity of the supply enzymes. (*B*) The narrowing of the population distribution shown after a single exposure to high glucose. The phenotypic plasticity of the population (range of phenotypes with higher than wild-type supply glycolytic activity) decreases at lower shunt activities ($C^{FBP}_{supply} > 0$) due to loss of FBP homeostasis. (*C*) The same as *B* but after five exposures to high glucose. Repeated exposures further narrow phenotypic plasticity.

states studied. During late fermentation, the minor isoforms are suppressed, consistent with their findings of no difference with wild type. During respiration, the major isoforms such as PK1 are rapidly inhibited and then broken down by posttranslational mechanisms (11, 23, 32), so that also no difference with wild type would be anticipated. The expression of the minor isoforms, such as HK1 and PK2, during respiration would also not impact metabolism, because they are not active in this state due to their low elasticities to glycolytic intermediates and covalent modifications. Due to the deletion of the minor isoforms a large reduction in glucose metabolism would be anticipated in early fermentation, until gene expression of the late fermentation dominant isoforms. However, their experimental design was not sensitive to metabolic differences in early fermentation due to relatively low time resolution and the major outcome measure being growth rate, which is suppressed for several hours after glucose exposure. Future studies combining engineered yeast with detailed higher time-resolution metabolic measurements could test this explanation as well as further elucidate the specific functional roles of enzyme isoforms.

The early and late fermentation phenotypes of the Crabtree effect coevolved ~100 million years ago via changes in a substantial number of regulatory elements that impact both the gene expression of metabolic enzymes and signaling pathways and duplications that led to the HK2, PFK2, PK1, and other isoforms that are expressed during respiration (35, 36, 45). Hypotheses for the advantages that led to selection of the Crabtree phenotype have focused on it reducing the time to adapt to glucose metabolism as well as increasing the rate of ETOH production (35, 36, 47). However, the role of phenotypic plasticity in the evolution of Crabtree positive yeast has not previously been addressed. We found that the homeostatic regulation provided by the glycogen/trehalose shunt could have allowed for greater phenotypic plasticity of glycolysis to exist in the yeast population. Increased phenotypic plasticity has been shown to provide a mechanism for enhanced selection by West-Eberhart (5) and Bateson (6), through allowing a portion of the population to survive environmental changes for a sufficient number of generations for genotypic alterations to enhance the adaptation already present in the phenotype.

We emphasize however that in the selection for Crabtree positive phenotypes, the metabolic response is precise and functional, whereas the whole-organism phenotypic plasticity induced by exposure to a new environment is initially not necessarily adapted to that change. The bundle of reaction norms expressed by the variable genotypes in the population can initially be quite scattered; it will only be honed by natural selection into more precise responses if the environmental changes are frequently encountered. Those reaction norms do, however, contain within their variation some responses that allow the population, if not every individual in it, to survive the change. The rapid changes possible in metabolism, which allow yeast with allelic substitutions to survive a glucose challenge, buy time for successive generations with the mutation to survive until a subsequent allelic substitution occurs that further enhances the glycolytic and ETOH production fluxes. We suggest that this latter process, at the level of the population, is the one that allowed the Crabtree Effect to originate and gave it time to be polished into its current state by evolution.

Although our findings are specific to the Crabtree effect, there are many examples of metabolic pathways that can rapidly switch from a low to high flux rate during challenges such as increased energy demand or substrate supply before a change in gene expression. Mammalian muscle and liver carbohydrate metabolism has been shown to rapidly respond to challenges during exercise or after glucose feeding (16–19, 30, 31). Even longer term adaptations can be mediated through post transcriptional mechanisms, for example the increase in mitochondrial oxidation capacity in aging mice due to regular exercise has been shown to be due to post translational mechanisms (19). Similarly, the large changes in gene expression after environmental changes in yeast

and other microorganisms have been paralleled by similar findings in multicellular organisms (1), which Fell originally suggested could play a role in metabolic homeostasis (48). We propose as a testable hypothesis that rather than being the primary cause of flux adaptation, these expression changes mainly provide homeostatic mechanisms that allow greater energetic efficiency and product formation.

The complexity of the relationships between genomics, phenotype, and the environment was envisaged by Evelyn Fox Keller, who wrote: "For almost fifty years, we lulled ourselves into believing that, in discovering the molecular basis of genetic information, we had found the secret of life; we were confident that if we could only decode the message in DNA's sequence of nucleotides, we would understand the "program" that makes an organism what it is. Furthermore, we marveled at how simple the answer seemed to be" (ref. 49, p. 8).

In exposing the consequences of oversimplifying the issues, Fox Keller finds common ground with West-Eberhard (5) and Bateson (6), who have written extensively about examples showing that phenotypic plasticity can enhance the range of responses to selection. Our findings are in accord with theirs by demonstrating that the complex interplay of metabolic and genomic homeostatic mechanisms link physiological adaptation within the lifetime of an individual to evolutionary adaptation over the course of generations by an increased plasticity of metabolic phenotypes.

Materials and Methods

Flux and Concentration Measurements. For all studies, yeast (*S. cerevisiae)* prior to the measurements were in kept under aerobic glucose-limited conditions in a medium containing acetate or ETOH as the main carbon fuel source. After the yeast were in the stationary state, they were then removed from this medium and studied in a medium containing high levels of glucose as the only carbon fuel source. The references from which we obtained flux and concentration measurements as the specific conditions the yeast were under are described below.

Respiratory state. The flux measurements were performed using ^{13}C NMR by replacing acetate in the media with [2-^{13}C]-labeled acetate (50, 51).

Early fermentation. Flux (52, 53) and concentration (53, 54) measurements were performed in a nitrogen-restricted medium containing a high concentration of [1-^{13}C]glucose under aerobic conditions. During these conditions, yeast in the stationary state rapidly synthesize glycogen and trehalose (55). Flux measurements were performed using ^{13}C and ^{31}P magnetic resonance spectroscopy (MRS), including measurements of futile cycling between F6P and FBP.

Late fermentation. Relative fluxes and concentrations between early and late fermentation were calculated from studies that measured them under both conditions. These ratios were then used to multiply the early fermentation values from the ^{13}C and ^{31}P MRS studies. Relative fluxes of glucose uptake and glycogen synthesis were estimated from the figures in the report by Becker et al. (55) for yeast studied under aerobic conditions using a high-glucose and nitrogen-restricted medium similar to that for the ^{13}C and ^{31}P MRS studies. The relative fluxes between early and late fermentation of the demand portion of glycolysis were determined from Hagman et al. (35, 36) under aerobic conditions in a high-glucose medium containing nitrogen sources. Although no glycogen was synthesized in these studies, as shown by Becker et al. (55), the availability of nitrogen does not impact the demand glycolytic flux and ETOH production. Relative F6P, G6P, FBP, T6P, and F2,6BP concentrations between early and late fermentation were obtained from van den Brink et al. (12) under high-glucose anaerobic conditions. Based on other studies (11, 42), the early-to-late concentration ratios would not be significantly impacted by the difference in O_2 levels (11, 42).

Enzyme activity and gene expression rates. Relative enzyme total activities, determined from V_{max} values, between early and late fermentation were obtained from van den Brink et al. (12), who comprehensively measured the V_{max} and isoform composition of all the enzymes in the glycolytic pathway. Although the measurements were performed under anaerobic conditions, only a small impact on glycolytic enzyme total activity is expected relative to aerobic conditions during late fermentation (11). Gene expression levels of glycolytic enzyme isoforms in late fermentation versus the respiratory/early fermentation state were from DeRisi et al. (9).

Data Availability. All study data are included in the article and *SI Appendix*.

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