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Decoupling Nutrient Signaling from Growth Rate Causes Aerobic Glycolysis and Deregulation of Cell Size and Gene Expression

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Running Title: Deregulation of Cell Growth and Division

Abbreviations: CDC - cell division cycle; GRR - growth rate response; UGRR - universal growth rate response; GRS - growth rate signal; TCA – tricarboxylic acid

Summary for TOC: The nutrition and the growth rate of a cell are two interacting factors with pervasive physiological effects. Our experiments decouple these factors and demonstrate the role of a growth rate signal, independent of the actual rate of biomass increase, on gene regulation, the cell division cycle, and the switch to a respiro-fermentative metabolism.

Abstract

To survive and proliferate, cells need to coordinate their metabolism, gene expression and cell division. To understand this coordination and the consequences of its failure, we uncoupled biomass synthesis from nutrient signaling by growing, in chemostats, yeast auxotrophs for histidine, lysine, or uracil in excess of natural nutrients (i.e., sources of carbon, nitrogen, sulfur and phosphorus), such that their growth rates were regulated by the availability of their auxotrophic requirements. The physiological and transcriptional responses to growth-rate changes of these cultures differed markedly from the respective responses of prototrophs whose growth-rate is regulated by the availability of natural nutrients. The data for all auxotrophs at all growth rates recapitulated the features of aerobic glycolysis, fermentation despite high oxygen levels in the growth media. In addition, we discovered very wide bimodal distributions of cell sizes, indicating a decoupling between the cell division cycle (CDC) and biomass production. The aerobic glycolysis was reflected in a general signature of anaerobic growth, including substantial reduction in the expression levels of mitochondrial and TCA genes. We also found that the magnitude of the transcriptional growth rate response in the auxotrophs is only 40-50% of the magnitude in prototrophs. Furthermore, the auxotrophic cultures express autophagy genes at substantially lower levels, which likely contributes to their lower viability. Our observations suggest that a growth rate signal, which is a function of the abundance of essential natural nutrients, regulates fermentation/respiration, the growth rate response, and the CDC.
Introduction

Regulating growth in diverse and fluctuating environments is essential for the survival of any organism. For a microorganism, the primary factors determining growth are natural nutrients that provide essential chemical elements and energy (Johnston et al, 1977; Hedbacker and Carlson, 2008; Broach, 2012). Yeast has evolved to detect the concentrations of such essential natural nutrients and transduce them into an appropriate growth rate response. This growth rate response involves a systems-level coordination of metabolism, gene expression and cell division that is similar for different nutrient limitations and sources of carbon and energy (Brauer et al, 2008; Slavov and Botstein, 2011, 2010). To understand better this coordination and the physiological consequences of its failure, we studied yeast cultures whose growth is limited by an auxotrophic requirement, a nutrient made necessary by a mutation.

Brauer et al (2008) discovered that leucine and uracil auxotrophs, whose growth is limited by their respective auxotrophic requirements (leucine or uracil), catabolize glucose through glycolysis to ethanol even in well oxygenated media (aerobic glycolysis) and fail to arrest their cell division cycle (CDC). Building upon this discovery, Boer et al (2008) extended the known differences between auxotrophic and natural limitations by measuring a 5 times faster decline in viability of auxotrophs starved for their auxotrophic requirements compared to prototrophs tarved for natural nutrients. Furthermore, Boer et al (2008) found that inactivating mutations in the target of rapamycin (TOR) network mitigate substantially the phenotypes of glucose wasting and decreased viability exhibited by auxotrophs limited on their auxotrophic requirement. These findings suggest a hypothesis, namely that the growth rate signal mediated by the TOR network, which normally signals nutrient sufficiency, is likely misregulated when cellular growth is limited only by an auxotrophic requirement and not by natural nutrients, such as the sources of carbon, nitrogen, sulfur or phosphorus. In other words, the cells are misled by the output of the TOR network and attempt to grow faster than allowed by the limiting concentration of the auxotrophic requirement.

Such misregulation by the TOR mediated growth rate signal raises the possibility that auxotrophs limited for their auxotrophic requirements might not be able to induce a wild type universal growth rate response as pro- totrophs do when cell growth is limited by a natural nutrient (Slavov and Botstein, 2011). In particular, auxotrophs may fail to induce genes specific to slow growth since they sense high concentrations of natural nutrients even at the slowest dilution rates when their growth is limited by an auxotrophic requirement. One may expect to see such failure in the leucine and uracil limited cultures grown by Brauer et al (2008). Because of the glucose wasting, however, those auxotrophic cultures were also at least partially limited on glucose as indicated by the very low residual concentration of glucose (below the limit of detection) in the culture media (Brauer et al, 2008). To measure the growth rate response in auxotrophs independent of glucose depletion, we conducted a series of growth rate experiments with histidine, lysine and uracil auxotrophs fed with media containing 2% glucose, 4 times more glucose than in the Brauer et al (2008) experiments. Like Brauer et al (2008), we observed glucose wasting in our experiments; however, the residual
glucose remained quite high in all cultures and at all growth rates, such that the cultures were limited only by their auxotrophic requirement and glucose remained in excess.

The results of these experiments showed that the cell size varies substantially in auxotrophs limited for their auxotrophic requirements, indicating a disconnect between biomass increase and cell division. We also found that in auxotrophs, the magnitude of induction of growth rate response genes is only 40 - 50% of the magnitude in prototrophs, signaling disregulation of the growth rate response. Furthermore, we found that genes known to be specific to aerobic growth (ter Linde et al. 1999) are expressed at very low levels in auxotrophs limited for their auxotrophic requirements. Such genes include most mitochondrial genes (especially the ones coding for mitochondrial ribosomal proteins), all Krebs and glyoxylate cycle genes including key dehydrogenases (IDP2, ADH1 and ADH2), and regulatory metabolic enzymes (ICLI, CIT1, and FOX2). Similarly, gene sets upregulated in anaerobic cultures were also upregulated in auxotrophs limited for their auxotrophic requirements, including CIT2, and seripauperin (DAN) genes. These findings help characterize the physiological and transcriptional mechanisms regulating the CDC, growth rate and respiration.

Results

Experimental Design

To explore how lysine and histidine auxotrophs adapt or fail to adapt their growth and metabolism to changes in the doubling time (growth rate) that is controlled by the levels of their auxotrophic requirements, we grew and characterized continuous cultures of his3 and lys2 non-reverting mutants. The amino acids histidine and lysine were chosen to expand the scope of profiled auxotrophs limited for their auxotrophic requirements. Also, histidine was chosen as a common condition with previous work (Boer et al, 2008). To avoid complications from glucose wasting, the chemostat feed media had 20g/L glucose to ensure excess residual glucose in the cultures even when the cultures waste glucose. The media were supplemented with the limiting concentrations of the auxotrophic requirements (amino acids); see Methods.

The growth rates were the same as in the experiments by Brauer et al (2008), \( \mu = \{0.05, 0.10, 0.15, 0.20, 0.25, 0.30\} \) h\(^{-1} \) with corresponding doubling times of the cultures \( \ln(2)/\mu = \{13.8, 6.9, 4.6, 3.5, 2.8, 2.3\} \) h. For each steady–state culture, we measured cell density, distribution of cell-sizes, residual glucose, generated ethanol, and gene expression.

Glucose Consumption and Ethanol Production

At slow dilution rates, the rate of influx of fresh media is low and the cells spend more time in the reaction vessels. In addition, the concentration of the limiting auxotrophic requirement in the chemostat vessel is low, resulting in stringent limitation, which is likely to cause more glucose wasting. All of these 3 factors suggest that the concentration of residual glucose should be inversely correlated to the growth rate of the cultures, which is in fact what was experimentally observed. Fig. 1A. The cultures have large specific consumption of glucose since they ferment it to ethanol Fig. 1B. However, the residual glucose concentration is high even at the slowest dilution rate (\( \mu = 0.05 \) h\(^{-1} \)), and thus the growth of the cultures is not likely to be limited by glucose. The same 3 factors that result in low concentration of glucose at slow growth rate suggest that the concentration of generated ethanol in the culture media
should be high at slow growth and decrease as growth rate increases. In agreement with this expectation, we measured monotonically decreasing ethanol concentrations with increase in growth rate, Fig. 1B. In contrast, Brauer et al (2008) measured increasing ethanol concentrations with increase in the growth rate of the cultures limited by leucine or uracil. This difference likely arises from glucose depletion in the leucine and uracil cultures grown by Brauer et al (2008), especially at the slowest growth rates. To test whether this is indeed the case, we measured ethanol in the uracil and leucine limited cultures grown by Boer et al (2010) with 22g/L glucose in the feed media. The results (Fig. 1C) indicate the expected trend: ethanol concentrations decrease monotonically with growth rate.

**Failure to Coordinate Biomass Increase and Cell Division**

The steady–state cell densities of all auxotrophs limited for their auxotrophic requirements (Fig. 1D) decrease with increasing growth rate, similar to the results for prototrophic cultures whose growth is limited by natural nutrients (Brauer et al, 2008) and consistent with theoretical expectations (Slavov and Botstein, 2010, 2011). In contrast to cell density, the cell–size distributions for all three studied auxotrophs (Fig. 2ABC) differ markedly from the cell– size distributions for prototrophic cultures whose growth is limited by natural nutrients; for comparison, Figure 2D shows the results for a glucose limited prototroph and more data across more conditions can be found in our previous publications (Brauer et al, 2008; Slavov and Botstein, 2011). The cell–size distributions for all auxotrophs and for most growth rates are distinctively bimodal and broader than the distributions for the natural limitations, Fig. 2. For all auxotrophs, the median cell sizes and the bimodality increase with the increase in steady-state growth rates. These growth rate trends likely reflect two factors. First, the mean residence time for a cell in the chemostat vessel is inversely proportional to the growth rate. Thus, the slower the growth rate, the longer the cells are exposed to abundant natural nutrients without adequate biomass synthesis and the more likely to divide without having reached a normal size. The second factor is that increasing the growth rate of the cultures increases the residual concentrations of the limiting auxotrophic requirements and thus decreases the severity of the limitation. Conversely, the lower the growth rate, the more severe the limitation and the larger the fraction of very small cells.

Next, we sought to test whether the failure to coordinate cell growth and division that we observed in continuous cultures, manifested by decreasing cell sizes by increased severity of the auxotrophic limitation, is also present in batch cultures of auxotrophs limited for their auxotrophic requirements and whether this failure is general to strains with different genetic background, Fig. 3. For batch growth, decreasing the growth rate and increasing the severity of the limitation correspond to increased time spent in the batch cultures. Given the low viability of starving auxotrophs measured by Boer et al (2008), we limited the time courses of batch growth to 4 days since a large fraction of the cells are likely to have lost viability in longer starvation experiments. The earlier time points correspond to the higher growth rates in the continuous cultures and the later time points (days 3 and 4) correspond to the low dilution rates in the continuous cultures. The trend in these batch experiments (Fig. 3), for both genetic backgrounds tested, is consistent with the trend from the chemostat experiments: As time progresses, the severity of the starvation increases and so does the fraction of very small cells. We conclude that, both in batch and in continuous cultures, sensing extracellular natural
nutrients is essential for the coordination of cell growth and division (Johnston et al, 1977; Jorgensen et al, 2002) and this coordination is substantially perturbed when growth-rate signaling is misregulated and decoupled from actual increase in biomass.

### Transcriptional Data

The gene expression data for the histidine and lysine limited cultures are clustered hierarchically together with the gene expression data from prototrophs whose growth is limited by glucose or ammonium, Fig. 4. The clustering pattern indicates that auxotrophs and prototrophs differ both in the mean expression levels of sets of genes and in the growth rate trends. The prototrophic limitations show a very pronounced growth rate trend as described by Brauer et al (2008) and Slavov and Botstein (2011). Strikingly, this trend is not as pronounced in the auxotrophic limitations. This observation is consistent with our hypothesis that the signalling networks mediating the growth rate response are activated by natural nutrients but may not be activated to the same extent by nutrients made necessary by mutations.

While the expression levels of most genes are similar between the two auxotrophs, there are significant differences in the mean expression levels of genes between the auxotrophic and prototrophic limitations, Fig. 4. The first cluster of such genes, expressed more highly in the prototrophs, is strongly enriched for mitochondrial genes, redox functions, and respiration. The repression of those genes in auxotrophs likely reflects the shift from respiration to fermentation (Brauer et al, 2008). The second cluster of differentially expressed genes, expressed more highly in the auxotrophs, is strongly enriched for genes from amino acid metabolism, biosynthesis, and generation of nucleotides and precursor metabolites. Amino acid related genes tend to be expressed most highly in the slowest growing auxotrophic cultures, which likely reflects the lower concentration of the limiting amino acids (higher severity of the limitation) at low dilution rates. The third large cluster of differentially expressed genes, expressed more highly in the prototrophs, is strongly enriched for autophagy and vacuolar genes, Fig. 4. This observation suggests that auxotrophs fail to induce the expression of autophagy genes to wild type levels even at slow growth, perhaps because they sense high concentrations of all the natural nutrients and are unable to transduce the low concentrations of auxotrophic requirements into growth rate regulatory responses.

### Quantifying the Growth Rate Response

To identify and quantify monotonic growth rate trends in the gene expression, we regressed the gene expression levels on the growth-rate and computed growth rate (GR) slopes (Brauer et al, 2008; Airoldi et al, 2009; Slavov and Botstein, 2011), see the methods for details. Applying this analysis to the growth rate responses of prototrophic cultures whose growth is limited by natural nutrients, we previously identified a core set of 1500 genes that either increase or decrease with growth rate, at steady-state, in a very similar way across all studied nutrient limitations and sources of carbon and energy (Slavov and Botstein, 2011). We termed this condition-independent response to changes in the steady-state growth rate “universal growth rate response (UGRR)”. Next we explore the extent to which the auxotrophic limitations
induce the UGRR by comparing the distributions of GR slopes for UGRR genes between cultures whose growth is limited either by auxotrophic requirements or by natural nutrients.

**Weaker Induction of the Universal Growth Rate Response**

Our hypothesis is that auxotrophs forced to grow slowly because of shortage of a nutrient made necessary by a mutation may not be able to induce sufficiently the genes that are highly expressed when growth is limited by natural nutrients, as observed in the universal growth rate response (UGRR). To test this hypothesis, we quantified the magnitude of the UGRR. The distributions of growth rate slopes for the prototrophic limitations (glucose and ammonium) are shown in Fig. 5A with genes having positive (red distribution with mean slope 4.5) and negative (green distribution with mean slope -5.0) UGRR. The corresponding distributions of slopes for the histidine and lysine auxotrophs show similar qualitative trends but with significantly smaller magnitudes, Fig. 5B. Some of the genes with positive UGRR have negative growth rate slopes and the mean GR slope is only 1.9. Similarly, genes with negative UGRR have weaker induction in the auxotrophs, with mean GR slope -1.9. The ratios of the mean GR slopes for the genes with positive and negative universal growth rate response in auxotrophs and prototrophs (1.9 and ) indicate that on average the induction of the universal growth rate response in auxotrophs is more than 2 fold weaker compared to the induction in prototrophs.

Autophagy and ribosomal genes are among the most significantly over-represented functional groups among the genes with universal growth rate response (Slavov and Botstein, 2011). However, not all ribosomal and au-tophagy genes have UGRR. To test the possibility that auxotrophs respond to growth rate changes by inducing a different set of ribosomal and autophagy genes, we compare the distributions of growth rate slopes for all genes annotated as autophagy or ribosomes by the gene ontology, Fig. 5C-E. The comparison indicates that the weaker induction of the UGRR is general to all autophagy and ribosomal genes. Furthermore, the induction of the UGRR (as quantified by the distributions of GR slopes of ribosomal and autophagy genes) is also weaker for the leucine and uracil limitations grown by Brauer et al (2008), suggesting that weaker induction of the UGRR may be general to auxotrophic limitations. The difference between the GR slope distributions of histidine and lysine limited cultures growing in 2% glucose and the corresponding distributions for leucine and uracil cultures growing in 0.5% glucose media is likely due to the fact that the leucine and uracil cultures were also in part limited on a natural nutrient, glucose.

These differences in the GR slopes between different types of growth conditions raise the question whether the GR slopes are similar within each type of growth conditions. In other words, is the magnitude of the UGRR (as measured by growth rate slopes) characteristic of the type of growth conditions (auxotrophic versus prototrophic limitations) or is it specific to each limiting nutrient (His versus Lys)? To answer this question, we focused on autophagy genes with negative UGRR, and two types of growth conditions: (i) Auxotrophic limitations for His and Lys in 2% glucose and (ii) Prototrophic limitations for glucose and ammonium.
Plotting the slope distributions of autophagy genes for the two types of growth conditions (Fig. 6A) recapitulated the results from Fig. 5.

Consistent with the up-regulated growth signal from the TOR network, autophagy genes have significantly smaller growth rate response in auxotrophs, Fig. 6A. The same difference in induction of autophagy genes at slow growth can be represented as a plot of rank ordered (sorted) slopes, Fig. 6B. We then compared the distribution of slopes within each type of growth condition, Fig. 6C. The distributions of slopes for the His and the Lys limitations are statistically indistinguishable from one another, as indicated by the very large probability (computed from a rank sum test) that they come from the same distribution. Similarly, the distributions of slopes for the glucose and the ammonium limitation are statistically indistinguishable from one another, reinforcing the conclusion that the magnitude of the growth rate response is the same within each type of growth conditions. In contrast, any of the auxotrophic limitations has significantly smaller growth rate response compared to any of the prototrophic limitations. The within-group similarity and between-group difference indicate that the magnitude of the UGRR is characteristic of the nature of the limitations, natural nutrient or auxotrophic requirement. The difference between the growth rate response of autophagy genes may be a reflection of the weaker induction of the autophagy genes at slow growth, Fig. 6D.

Next we applied the same type of growth rate slope comparison to the peroxisomal genes, one of the functional groups of genes with common negative growth rate response in glucose carbon source and positive growth rate response in ethanol carbon source (Brauer et al., 2008; Slavov and Botstein, 2011). The results (Fig. 7A) indicate the same trend of reduced growth rate induction in the auxotrophs. Similar to the autophagy genes, the growth rate response of peroxisomal genes is characteristic of the nature of the limitation (Fig. 7A) and may be attributed at least in part to reduced induction of peroxisomal genes in slowly growing ($\mu = 0.05 h^{-1}$) auxotrophs, Fig. 7B.

**Anaerobic Transcriptional Response**

Despite high oxygen levels (85 - 95% of the saturation level) in the growth media, the auxotrophic cultures fermented glucose to ethanol (Fig. 1), a phenomenon known as aerobic glycolysis. This aerobic glycolysis is also reflected in the transcriptional response as exemplified by the lower expression levels of mitochondrial genes found from the cluster analysis of the hierarchically clustered gene expression data, Fig. 4. We sought, if possible, to expand this anaerobic transcriptional signature by identifying and quantifying the expression of sets of genes whose transcriptional responses reflect the aerobic glycolysis. To quantify the difference in the expression of mitochondrial genes, we plotted the distributions of fold changes for (i) auxotrophs (our cultures limited for His and Lys) fed by media containing 2% glucose as a sole source of carbon and energy, for (ii) prototrophs (the cultures limited for glucose and ammonium) growing on glucose as a sole source of carbon and energy and for (iii) prototrophs (the cultures limited for ethanol and ammonium) growing on ethanol as a sole source of carbon and energy, Fig. 8AB. All sets of mitochondrial genes turned out to have the highest expression in ethanol carbonsource, lower in the prototrophs growing in glucose carbon source, and the lowest expression in the auxotrophs.
These differences in expression were greatest for the mitochondrial ribosomal proteins (Fig. 8A) that have UGRR (Slavov and Botstein, 2011) and were also found for other mitochondrial genes as exemplified in Fig. 8A with the mitochondrial envelope genes.

We explored the expression of other sets of genes for which ter Linde et al (1999) reported differential regulation between aerobic and anaerobic steady–state chemostat cultures. Genes which are expressed at higher levels in anaerobic cultures, such as the seripauperin (PAU) genes (Rachidi et al, 2002; Hickman et al, 2011), were also expressed at higher levels in the auxotrophs relative to the prototrophs, Fig. 9A. The seripauperin genes constitute a large family of genes with highly homologous sequences and induced in anaerobic conditions. Although the microarrays are optimized to minimize cross-hybridization, cross-hybridization could contribute to the detected signal. Figure 9A show that all PAU genes detected unambiguously by the microarrays, PAU1, PAU2, PAU3, PAU4, PAU5, PAU6, and PAU7 are induced in the auxotrophs relative to the prototrophs.

Conversely, genes which are expressed at lower levels in anaerobic cultures are also expressed at lower levels in the auxotrophs relative to the prototrophs. Such genes include metabolic enzymes and the HAP transcription factors, Fig. 9B. We also found that all genes from the tricarboxylic acid (TCA) cycle were strongly down-regulated in the auxotrophs, Fig. 9C. This down–regulation is reminiscent of the down–regulation of these genes in the first (fermentative) phase of the diauxic shift (Brauer et al, 2005). The down–regulation of TCA genes likely reflects a major shift toward fermentative metabolism in the auxotrophs, which was not seen in the prototrophs growing at the same growth rates, Fig. 9C. ter Linde et al (1999) found that the peroxisomal citrate synthase CIT2 is upregulated in anaerobic conditions and this gene is also upregulated in the auxotrophic limitations, Fig. 9C. Thus in addition to mitochondrial genes, we find that many other genes known to be regulated differently between aerobic and anaerobic cultures (ter Linde et al, 1999) are also expressed differentially between the auxotrophic and the prototrophic limitations, forming an extensive expression signature characteristic of anaerobic/fermentative growth and metabolism, Fig. 9. This expression signature is consistent with the report by Petti et al (2011) that stationary cultures of auxotrophs consume less oxygen than stationary cultures of prototrophs; it is harder to reconcile with the report by (Basso et al, 2010) that uracil auxotroph whose growth-rate ($\mu = 0.1h^{-1}$) is limited by uracil consumes two times more oxygen than the prototrophic strain whose growth rate is the same and limited by the carbon source.

Despite the many transcriptional similarities between the auxotrophs limited for their auxotrophic requirements and anaerobic cultures, there are some differences. For example, as shown in Fig. 9A, most ergosterol biosynthesis genes (ERG) are induced in the auxotrophs (especially ERG1, ERG2, ERG3, ERG7, ERG8, ERG11, ERG24, ERG25, ERG27) but some of these genes (such as ERG2, and ERG11) are down-regulated in anaerobic conditions by a Hap1 mediated repression (Hickman and Winston, 2007). This difference between the expression of ERG genes during aerobic and anaerobic glycolysis most likely reflects the effect of oxygen on Hap1 (Hickman and Winston, 2007) and the fact that oxygen is required for the biosynthesis of ergosterol. Thus, at least some regulatory mechanisms differ between aerobic and anaerobic glycolysis while many transcriptional responses are highly similar.
Discussion

Decoupling the Growth Rate Signal from the Growth Rate

The evidence described above strongly supports our hypothesis that growth under the limitation for an auxotrophic requirement decouples the growth rate signal, which in yeast is a function of the concentrations of natural nutrients present in the growth medium, from the actual biomass production, which is fundamentally limited by the auxotrophic requirement. Under these growth conditions, we found that the induction of the universal growth rate response (GRR) (Slavov and Botstein, 2011) is incomplete. This finding suggests that the GRR is at least partially a consequence of nutrient sensing that gets integrated into a common growth rate signal (GRS) that ultimately results in the induction of the universal GRR. The integration of the nutrient signals into a GRS – the signal inducing the GRR – likely involves the target of rapamycin (TOR) since mutations inactivating TOR signaling mitigate phenotypes (glucose wasting and reduced viability) resulting from the decoupling of growth and growth rate signalling (Boer et al, 2008).

The decoupling of the growth rate from the concentrations of natural nutrients allows us to distinguish between two possible mechanisms of transcriptional and growth rate regulation: (i) the sensing of extracellular nutrients and (ii) the sensing of the availability of intracellular metabolites and energy. Levy et al (2007); Levy and Barkai (2009) attempted to distinguish between these two mechanisms by using either the temporal sequence of adaptations to perturbations or a deletion mutant of ADH1 to decouple growth rate from nutrient sensing. Based on the results, Levy et al (2007) concluded that the gene expression is regulated by sensing external stimuli, i.e., mechanism (i). In contrast (Boer et al, 2010) measured hundreds of metabolites in continuous steady-state cultures limited on different nutrients and growing over a range of growth rates and used the data to identify intracellular metabolites limiting the growth rate for each condition, i.e., mechanism (ii). Our data are consistent with and support both mechanisms for regulation of gene expression and growth rate. The differences in the physiological behavior (fermentation versus respiration) and the gene expression patterns (including the substantially weaker growth rate response in auxotrophs) indicate that sensing extracellular natural nutrients (mechanism (i)) plays an important role in setting the growth rate and the appropriate gene expression response. Such sensing of glucose is extensively studied (Youk and Van Oudenaarden, 2009; Broach, 2012) and our observations that diverse natural nutrients and carbon sources induce a very similar growth rate response (Slavov and Botstein, 2011) suggest a general role for the sensing of variety of natural nutrients in setting the appropriate growth rate and gene levels. At the same time, the finding that most growth-rate-responsive genes in the auxotrophs limited for their auxotrophic requirements have qualitatively the same type of growth rate response as they do in prototrophs, even if quantitatively much weaker, supports mechanism (ii); internal regulatory feedback-loops that sense intracellular metabolites, such as amino acid sensing by the TOR, also play a role in regulating gene expression and the growth rate.

Viability and the Induction of Autophagy at Slow Growth
It has long been known that mutants deficient in autophagy have lower viability, and the induction of autophagy is required for preserving viability at slow growth (Tsukada and Ohsumi, 1993; Takeda et al, 2010). Recently Gresham et al (2010) conducted a genome-wide systems–level experiment demonstrating that the deletion of autophagy genes results in reduced viability during starvation. We found here that auxotrophs limited for their auxotrophic requirements express autophagy genes at significantly lower levels than prototrophs with the same doubling period, and this decrease likely contributes to the lower viability of auxotrophs limited for their auxotrophic requirements (Boer et al, 2008). More recently, Petti et al (2011) also found that survival during starvation is correlated with expression of autophagy genes. A possibility, reconciling all data, is that slowly growing auxotrophs fail to transmit a signal (likely the growth signal involving TOR) for nutrient shortage and thus fail to induce normal autophagy. The mediation of such a signal by the TOR signaling pathway may be one of the reasons why mutations in TOR signaling mitigate the loss of viability in starving auxotrophs (Boer et al, 2008).

The Growth Rate Signal Regulates Respiration and Fermentation

The decoupling of the growth rate signal from growth (biomass production) helps clarify the regulatory role of the growth rate signal in the balance between respiratory and fermentative growth. Brauer et al (2008) discovered that prototrophic cultures growing slowly because of the shortage of a natural nutrient respiate even when the residual glucose in the growth media is high. In stark contrast, auxotrophs limited for their auxotrophic requirements ferment at high rates at all growth rates, both in the experiments reported here and elsewhere (Brauer et al, 2008; Boer et al, 2008; Gresham et al, 2010). These results show that in all studied cases when the GRS is high (because of abundant natural nutrients), yeast catabolizes glucose primarily by fermentation. Conversely, in most studied cases when the GRS is low (because of the shortage of a natural nutrient), yeast catabolizes glucose primarily by oxidative phosphorylation. In addition to confirming the physiological phenotypes associated with strong GRS in auxotrophs, we find a very strong and clear transcriptional signal for anaerobic metabolism. The reduced expression of mitochondrial and TCA genes is an expression signature reminiscent of the first phase of diauxic growth (Brauer et al, 2005) and a clear signal for transcriptional regulation favoring fermentation and not respiration. This conclusion is further reinforced by an extensive anaerobic transcriptional signature; genes expressed highly in anaerobic cultures are also expressed highly in the auxotrophs limited for their auxotrophic requirements. Conversely, genes expressed at low levels in anaerobic cultures are also expressed at low levels in the auxotrophs limited for their auxotrophic requirements, Fig. 8 and Fig. 9.

We can generalize this regulatory link between the GRS and respiration/fermentation to higher eukaryotes by taking into account that in higher eukaryotes the GRS depends on growth factors. At low GRS, eukaryotic cells respire, with examples including regulated slow growth in late stage embryos and adult organisms. In contrast, at high GRS, eukaryotic cells ferment, with examples including early embryonic development, cell cultures growing in media containing excess of growth factors, and cancer cells with upregulated proliferative signals. The data in this paper and in the literature (Brand and Hermfisse, 1997b; Wise et al, 2008;
Heiden et al., 2001; Elstrom et al., 2004; Brauer et al., 2008; Boer et al., 2008; Heiden et al., 2009; Slavov and Dawson, 2009; Levine and Puzio-Kuter, 2010; Cairns et al., 2011; Dang, 2012) are all consistent with the possibility that the cellular regulatory networks are hardwired so that the strength of the GRS regulates the transition between respiratory and fermentative metabolisms, resulting in the observed correlation between aerobic glycolysis and rapid proliferation.

Importantly, however, the correlation between fast growth and aerobic glycolysis does not indicate that respiration, in principle, cannot sustain fast cell growth. To the contrary, some eukaryotes, including Crabtree negative yeasts, achieve fast cell growth without resorting to aerobic glycolysis (De Deken, 1966). Even budding yeast growing in glucose-limited chemostatic conditions can evolve a substantial reduction in the rate of aerobic glycolysis (Ferea et al., 1999; Gresham et al., 2008; Wenger et al., 2011). Rather, we suggest that the inefficient energy production per glucose molecule during aerobic glycolysis offers advantages to rapidly growing cells. The identity of such trade-offs has remained rather controversial for a century (Warburg et al., 1924; Warburg, 1962; Crabtree, 1929; Newsholme et al., 1985; Brand and Hermisffe, 1997a; Pfeiffer et al., 2001; Liu et al., 2010; Heiden et al., 2009; Levine and Puzio-Kuter, 2010; Shlomi et al., 2011; Dang, 2012). Our data support the hypothesis that the induction of aerobic glycolysis is regulated by the GRS and underscores the necessity for further experiments designed to directly identify the advantages of fermentation for fast-growing cells, and auxotrophs limited for their auxotrophic requirements may be a good model system for such experiments. Similarly, further work is necessary to investigate the mechanisms behind the striking similarity between the gene expression patterns of our auxotrophic cultures and cultures growing in anaerobic conditions. While the transcriptional responses are very similar, the mechanisms of gene-regulation in the presence and absence of oxygen may differ (Hickman and Winston, 2007; Hickman et al., 2011). Combining some of the transcriptional differences, such as the expression as ergosterol biosynthesis genes with network inference and other computational algorithms (Slavov, 2010; Tagkopoulos et al., 2005; Petti et al., 2011) can help identify regulators that differ between aerobic and anaerobic glycolysis. Similarly, it would be interesting to investigate whether the regulatory mechanisms involve chromatin remodeling (Rando and Winston, 2012).

Decoupling of Cell Division and Cell Growth

The large dispersion (variance) and bimodality in the distributions of cell sizes observed in all studied auxotrophs limited for their auxotrophic requirements suggest a decoupling between biomass production and cell division, Fig. 2 and Fig. 3. This result likely reflects decoupling between the cell division cycle and the growth cycle of biomass production (Slavov et al., 2011, 2012; Silverman et al., 2010), possibly resulting from the combination of a strong GRS and insufficient supply of the auxotrophic requirement. This decoupling appears to be a general phenomenon as we observed it in strains having different genetic backgrounds and growing under different auxotrophic limitations. The growth rate trend in the cell size distributions in Fig. 2, as well as in the time courses in Fig. 3, suggest that auxotrophs limited for their auxotrophic requirements not only continue budding as discovered by Saldanha et al (2004); Brauer et al (2008), but may also divide whenstarved for an auxotrophic requirement, thus circumventing the CDC cell–size control (Nurse, 1975; Johnston et al., 1977, 1979; Jorgensen et al., 2002) and resulting in cells with very small sizes. While the strong GRS sent by abundant nutrients may play a role in this decoupling too, the mechanistic connections are not clear. Furthermore, it is unclear whether the small cells (corresponding to the left mode of
the cell–size distribution) are as viable as the large cells (right mode). This disregulation of cell growth and cell division may be another factor contributing to the lower viability of auxotrophs limited for their auxotrophic requirements.

Methods

Cultures and Physiological Measurements

For the growth rate experiments we used non-reverting mutants DBY9496 (MATa his3-1, MAL2-8c with CEN.PK113 background), DBY919/GRF80 (MATa lys2-80) and DBY9492 (MATa ura3-52, MAL2-8c with CEN.PK113 background). Consistent with the genotypes of the strains, the expression values measured for HIS3 in the histidine limitation and for LYS2 in the lysine limitation are either missing (no transcript detected) or very low (more than 30 fold down regulated relative to the reference). All non limiting nutrient concentrations were the same as the ones used by (Saldanha et al, 2004; Brauer et al, 2005, 2008) with the exception of the glucose. The glucose concentration was 20g/L. All limiting concentrations were first determined as limiting for final biomass in batch growth and then confirmed to be limiting and within the linear response range in continuous cultures. The histidine limitation medium contained [His] = 4mg/L, the lysine limited medium contained [Lys] = 6mg/L and the uracil limited medium contained [Ura] = 5mg/L. Chemostats were established in 500mL fermenter vessels (Sixfors; Infors AG, Bottmingen, Switzerland) containing 250mL of culture volume, stirred at 400rpm, and aerated with humidified and filtered air. Chemostat cultures were inoculated, monitored and grown to steady state as described previously (Brauer et al, 2005). All cultures were monitored for changes in cell density and dissolved oxygen and grown until these parameters remained steady for at least 24h. The cell density, cell sizes and ethanol concentrations were measured as previously described by Slavov and Botstein (2011).

Measuring mRNA Levels

To measure RNA levels we sampled 10-30ml of steady–state culture and vacuum filtered the cells followed by immediate freezing in liquid nitrogen and then in a freezer at −80°C. RNA for microarray analysis was extracted by the acid–phenol–chloroform method. RNA was amplified and labeled using the Agilent low RNA input fluorescent linear amplification kit (P/N 5184-3523; Agilent Technologies, Palo Alto, CA). This method involves initial synthesis of cDNA by using a poly(T) primer attached to a T7 promoter. Labeled cRNA is subsequently synthesized using T7 RNA polymerase and either C3 or C5 UTP. Each C3-labeled experimental cRNA sample was mixed with the C5-labeled reference cRNA and hybridized for 17 h at 65°C to custom Agilent Yeast oligomicroarrays 8 × 15k having 8 microarrays per glass slide. Microarrays were washed, scanned with an Agilent DNA microarray scanner (Agilent Technologies), and the resulting TIF files processed using Agilent Feature Extraction Software version 7.5. Resulting microarray intensity data were submitted to the PUMA Database for archiving. The reference for the microarrays was also the same as the one used by Slavov and Botstein (2011): mRNA from a glucose limited culture of DBY10085 growing at a dilution rate \( \mu = 0.25h^{-1} \). To download and explore the data interactively, visit: http://genomics-pubs.princeton.edu/Aerobic_glycolysis/
Models

Similar to Brauer et al (2008); Slavov and Botstein (2011) we use an exponential phenomenological model to quantify the dependence between the mRNA level and the growth rate, $mRNA_i = b_i e^{a_i \mu}$. In semi–log space, such exponential model “appears” linear: $\log(mRNA_i) = \log(b_i) + a_i \mu$. Assuming Gaussian noise, the growth rate slope can be inferred by least-squares linear regression. All slopes throughout this paper are computed based on regression models minimizing the sum of squared residuals. As a measure of goodness of fit, we use the fraction of variance explained by the model, which for the $j^{th}$ gene is quantified by $R_j^2$:

$$R_j^2 = 1 - \frac{\sum_{i=1}^{N} (x_{ij} - f_{ij})^2}{\sum_{i=1}^{N} (x_{ij} - \bar{x}_i)^2}$$

In equation 1, $y_j$ is a vector of expression levels of the $j^{th}$ gene ($j^{th}$ column in $Y$), $\bar{y}_j$ is its mean expression level, $i$ is index enumerating the set of conditions $\alpha$ used in the model and $f_{ij}$ is the model predication for the $i^{th}$ condition and $j^{th}$ gene. To assess statistical significance, we use bootstrapped p values equal to the fraction of randomly permuted datasets having $R^2$ equal or greater than the $R^2$ of the data.

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Figure 1. Physiological Data. (A) Glucose and (B) Ethanol concentrations in the chemostat vessels of his3 and lys2 auxotrophs fermenting glucose to ethanol. (C) Ethanol concentrations in leu and ura auxotrophs grown by Boer et al (2010). (D) Cell density of his3 and lys2 auxotrophs.
Figure 2. Cell Size Distributions of Continuous Chemostat Cultures. (A) his3 auxotroph at growth rates: $\mu = 0.05, 0.10, 0.15, 0.20, 0.25, 0.30/h$; (B) lys2 auxotroph at growth rates: $\mu = 0.05, 0.10, 0.15, 0.20, 0.25, 0.30/h$; (C) ura auxotroph at growth rates: $\mu = 0.10, 0.23, 0.30/h$; (D) prototroph (DBY10085, isogenic to the the uracil auxotroph except for the ura mutation) growing at $\mu = 0.25/h$. 
Figure 3. Cell Size Distributions of Batch Cultures. Cell size distributions in batch cultures of his3 auxotrophs with CEN.PK background (DBY9496) (A) and with S288c background (DBY12029) (B). To display the full dynamical range, the data are shown on log2 scale so that bright red 10 corresponds to $2^{10} = 1024$ cells and deep blue 2 corresponds to $2^2 = 4$ cells.
Figure 4. Transcriptional Response. Hierarchically clustered gene expression data from our continuous cultures of his3 limited for His and lys2 limited for Lys (left set of columns) and from a prototroph limited limited either for glucose or for ammonium (right set of columns), (Brauer et al, 2008). The similarity metric used for clustering (non–centered, variance normalized correlations) is computed using all data shown in the clustergram. The data are displayed as fold changes, relative to the reference (a glucose-limited culture growing at $\mu = 0.25h^{-1}$), on a log2 scale.
Figure 5. Growth Rate Response of Auxotrophs and Prototrophs. Growth rate slopes of the genes with universal growth rate response in: glucose & nitrogen limitations (A); histidine and lysine limitations (B); Growth rate slopes of autophagy (green) and ribosomal (red) genes in: glucose & nitrogen limitations (C); leucine and uracil limitations (D); histidine and lysine limitations (E);
Figure 6. Growth Rate Slopes and Fold Changes of Autophagy genes in Auxotrophs and in Prototrophs. (A) Distributions of slopes for autophagy genes in auxotrophs and in prototrophs. (B) Rank orders of growth rate slopes for autophagy genes in auxotrophs and in prototrophs. The data from panel B is presented for each growth condition and strain separately. (C) The expression data for all strains is only for the slowest growth rate $\mu = 0.05h^{-1}$. The statistical significance of the difference between the distributions is quantified by a non-parametric test, rank sum-test.
Figure 7. Peroxisomal Genes. (A) Rank orders of growth rate slopes for peroxisomal genes in the auxotrophs and in the prototrophs. (B) Rank orders of fold changes for peroxisomal genes in the auxotrophs and in the prototrophs. The expression data for all strains is only for the slowest growth rate $\mu = 0.05 h^{-1}$. The statistical significance of the difference between the distributions on both panels is quantified by a non-parametric rank sum–test.
Figure 8. Down Regulation of Mitochondrial Genes. Distributions of log_2 fold changes for mitochondrial ribosomal protein genes (A) and for mitochondrial envelope genes (B). The statistical significance of the difference between the distributions on both panels is quantified by a rank sum-test.
Figure 9. Expression Signature of Fermentative Growth and Metabolism. (A) PAU and Ergosterol biosynthesis genes. (B) Genes down regulated in anaerobic conditions. (C) Enzymes participating in the Krebs and glyoxylate cycles. The left panels show the raw data and the right panels show zero-transformed data to enhance the visibility of the growth rate trends. The data are displayed as fold changes, relative to the reference (a glucose-limited culture growing at $\mu = 0.25\ h^{-1}$), on a log_2 scale.