Yeast cells can access distinct quiescent states

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We conducted a phenotypic, transcriptional, metabolic, and genetic analysis of quiescence in yeast induced by starvation of prototrophic cells for one of three essential nutrients (glucose, nitrogen, or phosphate) and compared those results with those obtained with cells growing slowly due to nutrient limitation. These studies address two related questions: (1) Is quiescence a state distinct from any attained during mitotic growth, and (2) does the nature of quiescence differ depending on the means by which it is induced? We found that either limitation or starvation for any of the three nutrients elicits all of the physiological properties associated with quiescence, such as enhanced cell wall integrity and resistance to heat shock and oxidative stress. Moreover, the starvations result in a common transcriptional program, which is in large part a direct extrapolation of the changes that occur during slow growth. In contrast, the metabolic changes that occur upon starvation and the genetic requirements for surviving starvation differ significantly depending on the nutrient for which the cell is starved. The genes needed by cells to survive starvation do not overlap the genes that are induced upon starvation. We conclude that cells do not access a unique and discrete G₀ state, but rather are programmed, when nutrients are scarce, to prepare for a range of possible future stressors. Moreover, these survival strategies are not unique to quiescence, but are engaged by the cell in proportion to nutrient scarcity.

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Most living cells spend the vast majority of their lifetime in a quiescent, nongrowing state. In response to external stress, growth inhibitory signals, or starvation for one or more nutrients, cells exit the mitotic cycle and enter a poorly defined quiescent, or G₀, state (Fabrizio and Longo 2003; Gray et al. 2004; Collier et al. 2006). Investigators have ascribed to G₀ yeast cells a number of distinguishing characteristics, including enhanced resistance to heat and high osmolarity, substantially reduced translation, a specific transcriptional profile, increased levels of storage carbohydrates, and a thickened cell wall (Gray et al. 2004; Smets et al. 2010). However, the only unequivocal defining characteristic of quiescent cells is their ability to maintain viability under the growth-arrested condition and to resume mitotic growth once growth-promoting conditions are restored (Collier et al. 2006). This characteristic distinguishes quiescent fibroblasts or lymphocytes from terminally differentiated cells, which, while capable of maintaining

viability, are not readily reactivated for growth. Similarly, this characteristic distinguishes yeast cells suffering from “natural” starvation—for a carbon source, for instance—from those subject to an “unnatural starvation,” such as auxotrophic cells deprived of the required amino acid. In the latter case, cells rapidly lose viability (Saldanha et al. 2004; Boer et al. 2008; Brauer et al. 2008). Thus, the quiescent G₀ state requires the coordinated and deliberate deactivation of cells in response to a growth inhibitory signal or the impending depletion of a core nutrient and not simply the cessation of growth attendant on abrogation of protein or RNA synthesis.

Most studies on quiescence in yeast have examined stationary-phase cells; that is, cells grown to saturation in rich, glucose-containing medium (Gray et al. 2004). Such cells first deplete glucose, and then the residual ethanol in the culture that accumulated from the prior fermentation of glucose, and then enter quiescence. Less information is available on the nature of cells that enter quiescence owing to depletion of other nutrients, such as nitrogen or phosphate. In fact, most of the studies on stationary-phase cells are conducted with auxotrophic strains, precluding cross-studies of these strains on nitrogen starvation.

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Accordingly, previous work does not address whether cells starved for one nutrient enter the same G_0 state as those starved for a different nutrient. Transcriptional profiling indicates that starved cells induce a common set of stress-responsive genes regardless of which nutrient is limiting. However, cells also exhibit nutrient-specific alterations in gene expression upon starvation (Gasch et al. 2000). Moreover, glucose addition alone stimulates stationary-phase cells to exit quiescence (Granot and Snyder 1991), while addition of glucose clearly does not stimulate cells arrested by nitrogen starvation to resume growth. So, from these perspectives, all quiescent states are not the same.

A second unresolved issue is whether the attributes of quiescent yeast cells, or eukaryotic cells in general, arise as a consequence of entering a discrete developmental state or of simply growing very slowly. That is, do quiescent cells attain a state that is not normally attained during mitotic growth, or is quiescence simply a limit extrapolation of slow growth? Recent studies on global transcriptional profiles of yeast cells as a function of growth rate demonstrated that levels of a substantial subset of transcripts are highly correlated with growth rate, with one group directly proportional and a second group inversely proportional (Brauer et al. 2008; Airolidi et al. 2009). In fact, measurement of the transcript levels of this set of genes provides a highly accurate predictor of the growth rate of the cells at the time the transcripts were isolated. This set of growth rate-dependent genes significantly overlaps those genes associated with the environmental stress responses; that is, genes whose expression is acutely elevated or depressed as a consequence of any of a large variety of stresses, including nutrient depletion. Thus, one interpretation of this correlation is that many transcriptional changes previously associated with stationary-phase cells may simply be the extrapolation of transcriptional regulation in response to growth rate. Similarly, studies have shown that heat-shock resistance is inversely proportional to growth rate: Slower-growing cells are more resistant to transient heat shock than are faster growing cells (Elliott and Futcher 1993; Lu et al. 2009). Thus, the heat-shock resistance of the stationary phase does not appear to be a distinguishing property of quiescent cells, but simply the extrapolation of a property dependent on growth rate.

To address the nature of quiescence in yeast, we conducted a transcriptional, metabolic, and genetic analysis of yeast cells induced into quiescence by a variety of nutrient depletions. The results of this analysis indicate that, while starvation for any of the natural nutrients for yeast induces a common set of physiological properties and transcriptional responses, the metabolic programs and genetic requirements of quiescence are highly nutrient-specific. Moreover, even those common responses that yeast cells exhibit under all starvation conditions are, for the most part, straightforward extrapolations of the properties of slow-growing cells. Thus, yeast cells do not access a discrete G_0 quiescent state under conditions of starvation, but rather, as nutrient conditions decline, acquire stress resistance that enables survival of the different metabolic challenges caused by starvation for different nutrients. These properties also prepare cells for other

environmental insults, although the actual means by which cells effectively weather those insults differ for the different stresses.

Results

Physiological properties of quiescent yeast cells are extensions of those arising from slow growth

To determine the core attributes of quiescent yeast cells and the extent to which those attributes are extensions of slow growth, we examined a number of properties and characteristics of cells induced into quiescence by starvation for different nutrients and compared those with cells growing slowly due to limitation of the same nutrient. For all of our starvation experiments, prototrophic cells exponentially growing on synthetic media (SD) without added amino acids were harvested by filtration onto nylon filters, which were then placed on agarose plates containing SD limited for the nutrient for which the cells were subsequently starved. After 2.5 h of incubation at 30°C, the filters were transferred to agarose plates containing synthetic media lacking glucose, nitrogen, or phosphate (Brauer et al. 2006). By measuring the plating efficiency of cells maintained for increasing lengths of time on starvation plates, we determined that cells retained a significantly high level of viability for extended periods of starvation regardless of the nutrient for which they were starved (Fig. 1A). Viability at 8 d ranged from 40% for nitrogen starvation to 80% for glucose starvation. This compares with an observed viability of 0.01% after similarly starving a *ura3* strain for uracil (Boer et al. 2008). Moreover, as seen in Figure 1B, cells were distributed among G_1 , S, and G_2 phases of the cell cycle prior to starvation, but within 24 h of transfer to starvation media, >98% of the population accumulated with a 1n DNA content, regardless of the starvation condition. Finally, we determined the resistance to heat shock of cells starved for 4 d for any one of the nutrients. As evident in Figure 1C, regardless of the nutrient for which cells were starved, starved cells were substantially more resistant to heat shock than exponentially growing cells. Thus, by any of the standard criteria, prototrophic cells starved for a “natural” nutrient attain a quiescent state regardless of the nutrient for which they are starved.

Lu et al. (2009) showed recently that heat-shock sensitivity was correlated with growth rate: Slow-growing cells exhibit heat-shock resistance intermediate between that of exponential cells and cells arrested by phosphate starvation, confirming an earlier report (Elliott and Futcher 1993). To determine whether other properties ascribed to quiescent cells are also manifest during slow growth, we compared the sensitivity to oxidative stress and to zymolyase treatment of starved cells versus that of cells growing at different growth rates. To do so, we established nine cultures of our prototrophic strain in chemostats limited for glucose, nitrogen, or phosphate at three different nutrient dilution rates, as well as batch cultures of the same strain starved for 4 d for each of the nutrients. We then tested samples of each of the cultures for sensitivity

Figure 1. Physiological properties of quiescent cells are extensions of those of slow-growing cells. (A) Survival of strain Y3358 grown to exponential phase in limiting SD and transferred at time 0 to SD lacking glucose, nitrogen, or phosphate for the indicated amount of time. Survival of an isogenic *ura3* strain starved for uracil is also shown. Viability was determined by colony-forming units and normalized to the viability at the onset of starvation. (B) FACS analysis of Sytox Green-stained cells cultured in the same conditions as in A at the indicated times after transfer to starvation conditions. (C) Heat-shock sensitivity. Cells growing exponentially in limiting SD (left) or quiescent cells after 4 d of starvation for glucose, nitrogen, or phosphate (right) were incubated for the indicated times at 50°C or 53°C as indicated. Serial 10-fold dilutions were spotted onto YEPD plates and growth is shown after 2 d at 30°C. (D) Sensitivity to oxidative stress. Cells were grown to exponential phase in limiting SD, starved for 4 d for the indicated nutrient, or grown in chemostat at indicated growth rates. Aliquots were treated with 0 mM, 1 mM, 5 mM, 10 mM, or 50 mM H_2O_2 for 1 h at 30°C, and then 10-fold serial dilutions were spotted onto YEPD plates. Growth is shown after 2 d of incubation at 30°C. (E) Sensitivity to zymolyase. Cells were grown as in D. Cells from the culture samples were harvested and resuspended in 50 mM potassium phosphate (pH 7.5) to an OD (600 nm) of 0.5, and the indicated amount of zymolyase T20 solution (10 mg/mL) was added to a total volume of 1 mL per sample. ODs were determined after incubation for 40 min at 30°C.

to different levels of hydrogen peroxide. As shown in Figure 1D, exponentially growing cells are significantly more sensitive to this oxidative stress than are starved

cells, regardless of the nutrient for which they are starved. Slow-growing cells exhibit a level of sensitivity intermediate between those of exponential and starved cells, with

resistance increasing with a slower growth rate. Thus, as with heat-shock sensitivity, the response of quiescent cells to oxidative stress can be extrapolated from their response at intermediate growth rates.

The cell walls of stationary-phase cells are more resistant than those of exponentially growing cells to digestion by zymolyase, a complex mixture of proteases and β -1,3-glucanases, due to increased cross-linking or increased mass of the cell wall or both. As shown in Figure 1E, the resistance of cells to zymolyase increases with decreasing growth rate for all nutrient limitations: the fastest-growing cells being most sensitive and the slowest-growing cells being most resistant, confirming results from a previous study (Elliott and Fitcher 1993). Moreover, nitrogen- and phosphate-starved cells are as resistant, or more so, than the slowest-growing culture. Somewhat surprisingly, glucose-starved cells are relatively sensitive to zymolyase treatment, even though stationary-phase cells and cells growing slowly due to glucose limitation are quite resistant and even though glucose-starved cells are resistant to heat shock and oxidative stress, as noted above. This suggests that our glucose starvation regimen is not equivalent to growth of cells to saturation in rich medium, and, moreover, that a carbon source may be required for the synthesis or modification of the cell wall necessary to render slow-growing or starved cells resistant to digestion. In sum, these results demonstrate that zymolyase resistance, like resistance to oxidative stress and heat shock, is a property acquired in proportion to the doubling time of cells and is not a distinguishing characteristic of quiescence per se.

Transcriptional profiling of starved yeast cells reveals a limited set of genes with quiescence-specific expression

To assess the extent to which yeast cells exhibit a quiescence-specific transcriptional program, we examined the transcriptional profiles of cells over 8 d, following starvation for carbon, nitrogen, or phosphate. As evident from the heat map of the transcriptional profiles as a function of time of starvation (Fig. 2), the changes in transcription during starvation are, to a first approximation, independent of the depleted nutrient, consistent with previous observations (Gasch et al. 2000). At early time points, cells subjected to starvation, particularly for glucose and nitrogen, exhibit an acute transcriptional response, noticeably in repression of ribosomal protein and ribosomal biogenesis genes and induction of autophagy- and stress-responsive genes. However, by 24 h, all cells attain a transcriptional profile that remains basically unchanged for the remainder of the starvation period.

Previous studies revealed that a large fraction of the transcriptome responds primarily to growth rate, independent of the nutrient that limits growth. In order to determine the extent to which the pattern of expression under starvation is simply an extension of the patterns attained during slow growth, we compared our results with previous measurements of gene expression under nutrient limitation in chemostats (Fig. 2; Brauer et al. 2008). Since our transcriptional data for quiescent cells

were obtained using strain W303, and the prior growth limitation studies were conducted with strain CEN.PK, we repeated the quiescent transcription studies using strain CEN.PK as well, and then analyzed both the W303 and CEN.PK data. We constructed a linear model of gene expression changes that included both a growth rate component and a quiescence-specific term (Supplemental Fig. S1). We used this model to identify genes whose expression in quiescence either matched or deviated from the extrapolated growth rate trend, assuming that the growth rate in quiescent cells is equal to 0. At a false discovery rate (FDR) of 5%, we identified ~140 genes whose expression during quiescence was a direct extrapolation of the expression levels at a decreasing growth rate (Supplemental Table S3). These were divided essentially equally between those that increase expression and those that decrease expression upon starvation. We also identified a group of genes that exhibited both changes in expression as a function of growth rate and of starvation, but whose expression level in starvation was not completely accounted for by extrapolation of the growth rate effects. We further identified a significant number of genes exhibiting nutrient-specific starvation responses; that is, genes whose expression was elevated or repressed upon starvation for one nutrient but not the other two, and was independent of growth rate. Finally, we identified a significant number of genes that were induced (82 genes) or repressed (190 genes) at least two-fold in both strains upon starvation for every nutrient but did not exhibit a growth rate-specific pattern of expression (Supplemental Table S3). These genes are candidates for a quiescence-specific transcription program.

The collection of quiescence-specific genes whose mRNA levels are independent of growth rate encompasses a functionally distinct class from those with growth rate-correlated transcript levels. Those growth rate-regulated transcripts whose levels fall upon nutrient limitation and starvation include genes involved in cellular biosynthesis, particularly ribosome biogenesis, and those whose levels rise are enriched for genes involved in autophagy and the stress response. In contrast, those whose increased transcript levels in quiescence are independent of growth rate were enriched for transition metal ion transport (FDR corrected $P = 1.8 \times 10^{-3}$), membrane lipid biosynthesis ($P = 4.3 \times 10^{-4}$), protein modification ($P = 1.2 \times 10^{-5}$), and response to toxin ($P = 3.0 \times 10^{-5}$). Those whose decreased transcript levels in quiescence are independent of growth rate are enriched for cytokinesis ($P = 1.7 \times 10^{-17}$), chromosome organization and biogenesis ($P = 1.2 \times 10^{-12}$), and organization of the nuclear pore complex ($P = 2.3 \times 10^{-6}$). As evident from these categories, a significant number of the genes with reduced transcript levels participate in cell cycle processes and, although they are not growth rate-responsive, would be expected to not be required in the absence of growth. In fact, approximately a third of the quiescence-specific repressed genes are also repressed rapidly following heat shock (Supplemental Fig. S2). In sum, these results reveal a discrete but limited number of genes whose expression appears to be quiescence-specific.

Figure 2. Cells exhibit a limited quiescent-specific transcriptional response. (A) Heat map of the global transcriptional changes resulting from starvation for glucose (C), nitrogen (N), or phosphate (P) for increasing time, juxtaposed next to those resulting from growth at increasing doubling times. Data are shown for ~5500 genes that are organized by unsupervised hierarchical clustering. Data for expression as a function of nutrient limitation were taken from Brauer et al. (2008) for strain Y3840 with dilution rates of 0.05 h⁻¹, 0.1 h⁻¹, 0.15 h⁻¹, 0.2 h⁻¹, 0.25 h⁻¹, and 0.3 h⁻¹ (left to right). Reference for all chemostat samples is from a glucose-limited culture with a dilution rate of 0.25 h⁻¹. Starvation data were obtained with strain Y3358 starved for 30 min, 90 min, 270 min, 1 d, 2 d, 3 d, 4 d, or 8 d (left to right), normalized as described in the Materials and Methods to the reference culture in Brauer et al. (2008). Bars immediately to the left of the data identify those genes that show either growth rate-specific gene expression (green) or quiescence-specific gene expression over all three nutrient conditions. (B) Expression patterns of genes showing growth rate-specific expression (left), quiescence expression (middle), or both (right) are organized as in A.

To further probe the relationship of growth rate-specific expression to quiescent-specific expression, we analyzed the transcriptional data by singular value decomposition (SVD), an unsupervised approach for identifying independent patterns underlying data matrices (Supplemental Fig. S3). The strongest expression pattern, or “eigengene,” which accounts for 24% of the variation, corresponds to a growth rate-dependent pattern that extends into quiescence. The second eigengene, which explains 13% of the signal, captures a signal differentiating the nutrient conditions, with an interaction between carbon limitation and quiescence. The third eigengene, explaining 10% of the signal, corresponds to a change in expression during quiescence in all three nutrient conditions that is, in all cases, not an extrapolation of growth rate changes. This represents a quiescent-specific component of the transcriptional program. Thus, as with the analysis above, SVD suggests that a discrete but limited transcriptional program underlies quiescence.

Different starvation regimens yield different metabolic profiles

Using LC-MS/MS, we determined quantitative trends for ~100 compounds over the course of nutrient starvation, obtaining measurements for most central carbon metabolites, amino acids, and nucleotides (Supplemental Table S4). We combined these metabolic profiles of starving cells with those obtained previously for cells growing under limited nutrients and organized the data by hierarchical clustering (Fig. 3). As evident from these data, cells exhibit an acute response in their metabolic profiles upon transfer to starvation media, but attain within 24 h a pattern that remains essentially unchanged for the duration of the starvation period. This time course is similar to that observed for transcriptional changes following starvation, suggesting that cells attain a stable quiescent program within a day of starvation. However, unlike the transcriptional profile following starvation, the metabolic profile

Figure 3. Metabolic profiles differ during starvation for different nutrients. Relative metabolite levels in nutrient-starved and nutrient-limited cultures were obtained as described in the Materials and Methods and are represented as a heat map, organized as in Figure 2A. The reference for all samples is from an exponential culture growing on SD.

differs substantially depending on the nutrient for which the culture was starved. This observation is consistent with those of previous studies on the metabolic profiles of yeast cells as a function of limiting nutrients, which revealed that metabolic patterns were quite dependent on which nutrient was limiting and that few metabolites showed growth rate-dependent changes uniformly across all starvation conditions (Boer et al. 2010).

The metabolic profiles obtained upon starvation can most readily be explained through mass action as either the depletion of those internal compounds most closely related to the eliminated nutrient or the accumulation of intermediates in a pathway due to lack of subsequent reaction partners. For instance, most amino acids are depleted in nitrogen-starved cells but not in glucose- or phosphate-starved cells, with the attendant accumulation of α-ketoglutarate. Similarly, nucleotide triphosphates are depleted in phosphate-starved but not nitrogen-starved cells, with an attendant increase in nucleosides, their dephosphorylated degradation products. In addition, TCA cycle intermediates accumulate in nitrogen- and phosphate-starved cells

but not in glucose-starved cells. In contrast to these overall trends, glycolytic intermediate levels show divergent behavior in glucose starvation: Fructose bisphosphate declines while phosphoenolpyruvate increases. This can be rationalized based on turning off of pyruvate kinase, combined with a shift in cofactor concentrations that thermodynamically favors accumulation of the trapped intermediates in more downstream compounds. Specifically, the observed rise in ADP/ATP and NAD⁺/NADH ratios favors carbon residing downstream from phosphoglycerate kinase. Thus, the levels of most metabolites during starvation appear to reflect mass action, and, accordingly, few if any of the measured metabolites show the same behavior over all starvation conditions.

As with transcript levels, some of the metabolite levels attained on starvation for a particular nutrient are direct extrapolations of those obtained from limitation for that nutrient. For instance, the depletion of amino acids and accumulation of TCA cycle intermediates in nitrogen starvation can be extrapolated from the levels of those compounds in cells grown under nitrogen limitation. The

metabolic intermediates of glycolysis—hexose-phosphate, fructose-1,6-bisphosphate, dihydroxyacetone phosphate, 2,3-diphosphoglycerate, and pyruvate—were depleted during starvation for all nutrients, albeit to a substantially greater extent in glucose and phosphate starvation than in nitrogen starvation. Most of these metabolites also exhibited a growth rate-dependent decline under all limitations. The decline in these metabolites in glucose-limited cells can be attributed to the absence of fermentable sugar, while the decline in phosphate-limited cells likely results in part from the absence of phosphate needed for formation of the high-energy phosphate bonds during glycolysis. However, the decline observed in nitrogen limitation implies that suppression of glycolytic activity constitutes a more general response to diminished growth conditions, driven also by signaling events.

In contrast to these examples, several metabolites show discordance in their levels attained during starvation relative to their levels under limiting nutrient conditions. For instance, levels of nucleosides and bases decline with decreasing growth rate in limiting nitrogen, but increase substantially during starvation for nitrogen. This may result from rRNA degradation following autophagic degradation of ribosomes under this starvation condition. Similarly, TCA cycle intermediates increase with decreasing growth rate on limiting glucose but decline during glucose starvation, presumably reflecting the paucity of substrates entering the cycle. In sum, as with transcript levels, some metabolite levels in starvation extrapolate from those attained during slow growth, while others show marked divergence from that extrapolation. However, unlike transcript levels, none of the compounds shows a starvation-specific behavior over all starvation conditions.

Trehalose accumulates in stationary-phase cells and is thought to provide protection against various stresses in the quiescent state (Gray et al. 2004; Shi et al. 2010). Moreover, trehalose is one of the few metabolites whose levels are anti-correlated with growth rate over all growth-limiting conditions examined (Boer et al. 2010). These observations are consistent with trehalose synthesis occurring predominantly during the G_1 phase of the cell cycle, which expands as cells grow slower. Moreover, trehalose accumulation during stationary phase or G_1 provides a reservoir of fermentable sugar to drive nutrient-limited or previously starved cells through “Start” to initiate the cell cycle. Somewhat surprisingly, trehalose levels under starvation were reduced relative to the value extrapolated from the growth rate trend (Fig. 4). While nitrogen-starved cells do accumulate trehalose, the levels do not reach those projected based on growth rate. Cells starved for glucose initially deplete trehalose and, although the concentration of trehalose subsequently increases, it never returns to a prestarvation level. Thus, in this respect as well, glucose-starved cells do not behave like stationary-phase cells, in which trehalose levels increase significantly. Cells rapidly deplete trehalose upon phosphate starvation and the levels remain low throughout quiescence. This likely results from depletion under this condition of the substrates for trehalose synthesis (glucose-6-phosphate and UDP-glucose), both of which

Figure 4. Trehalose metabolism in starved cells. Relative trehalose levels (*top* panel) and *TPS2* mRNA levels (*bottom* panel) are shown following starvation for glucose, nitrogen, or phosphate, and as a function of dilution rate in chemostats limiting for those nutrients. The reference for all samples is from an exponential culture growing on SD. The data for starvation and limitation in each panel are plotted using the same vertical axis scale

rapidly decline following phosphate starvation. Finally, despite the reduction in trehalose levels during starvation, phosphate-starved and glucose-starved cells maintain viability and exhibit stress-resistant phenotypes equivalent to those of stationary-phase cells. Accordingly, cells do not require trehalose as a stress protectant under these quiescent conditions.

Correlation of metabolism and transcription during quiescence

In order to explore the interplay between transcription and metabolism, particularly as it occurs during quiescence, we developed a visualization program that enables a comprehensive view of metabolism and integrates mRNA transcript abundance information in concert with metabolite concentration (<http://genomics-pubs.princeton.edu/quiescence/VPAD>). This program, which we call VPAD, uses the JavaScript library “Raphaël” to map metabolite concentrations and mRNA abundance values as colors onto a megapathway of yeast metabolism that spans the biosynthetic production of nucleotide triphosphates, amino acids, cofactors, reserve, and central carbon metabolism. The visualization is readily shared and accessed through an internet browser that facilitates navigation through large data sets. This program provides a framework for analyzing transcriptional or other global data as it relates to metabolic changes.

The coincident visualization afforded by VPAD of metabolite and transcripts levels highlights the uncorrelated behavior of transcripts and metabolites during

starvation. Two examples clearly demonstrate this lack of correlation. After 4 d of starvation for any of the nutrients, transcript levels for the trehalose biosynthetic enzymes—trehalose-6-phosphate synthase and trehalose-6-phosphate phosphatase—are substantially elevated relative to those in exponentially growing cells, as are the transcripts for the two neutral trehalases (Fig. 4; Supplemental Fig. S4). However, under nitrogen starvation, trehalose levels are quite high, whereas under glucose and phosphate starvation, trehalose levels are unchanged or even reduced relative to those in exponentially growing cells. Thus, there is no correlation between trehalose levels and the levels of transcripts encoding enzymes responsible for synthesis and degradation of the metabolite. Similarly, while transcript levels of TCA and glyoxylate cycle genes responded in various ways to the effects of starvation, none of the genes exhibited a nutrient-specific variation, with the exception of the transcripts for citrate synthase, which were elevated in glucose starvation but not in phosphate or nitrogen starvation. In contrast, the levels of TCA intermediates were substantially decreased in glucose-starved cells, substantially elevated in nitrogen-starved cells, and either elevated or unchanged in phosphate-starved cells (Fig. 5). Thus, transcript levels do not dictate metabolite levels. Rather, we conclude that the levels of metabolic enzyme, along with their post-translational modifications and allosteric regulation, coupled to metabolite mass action, determine the levels of metabolic intermediates in the cell. In short, transcript levels reflect what the cell would like to do, while the metabolite levels reflect what it can do.

Different starvations impose different genetic requirements for survival

In order to clarify further whether quiescent states induced by different nutrient starvations have common or distinct properties, we conducted a genome-wide screen of the nonessential, prototrophic deletion collection for mutants defective in survival during quiescence induced by different starvation conditions. We conducted the screen in two different ways. In the first screen, the array of individual deletions was grown on SD plates, then transferred to SD-limiting plates (see the Materials and Methods), and finally onto plates lacking either glucose, nitrogen, or phosphate. After 9 d of incubation at 30°C, the arrays were pinned onto YEPD plates, and those strains that failed to grow after 2 d were recorded.

To confirm and quantify the results obtained in this initial screen, we conducted a second screen in which the pooled deletion strains were starved in liquid and the survivors were detected by sequencing methods. Specifically, the deletion collection was pooled and grown to exponential phase in complete or limiting YNB liquid medium. A sample from each culture was spread on multiple YEPD plates to yield $\sim 2 \times 10^6$ distinct colonies, and the remainder of the culture was transferred to the appropriate starvation culture medium. After incubation in the liquid culture for 9 d at 30°C, a sample from each culture was spread on multiple YEPD plates to

Figure 5. Discordance in TCA cycle intermediates and gene expression levels during starvation. On a diagram of the TCA cycle consisting of the TCA cycle intermediates (ovals) and the genes encoding enzymes (rounded rectangles) catalyzing interconversion of those intermediates are indicated the change in levels of the metabolites and mRNAs at 4 d of starvation for glucose (*top panel*), nitrogen (*middle panel*), or phosphate (*bottom panel*) relative to those in cells growing exponentially on SD.

yield $\sim 2 \times 10^6$ distinct colonies. The colonies from each sample were pooled, genomic DNA was extracted, and the UP and DOWN tags were amplified by PCR. The pooled PCR reaction was sequenced on an Illumina sequencer from which $>10^7$ sequences were recovered, 70% of which could be assigned to one of ~ 3500 deletion strains. The number of times the sequence for a particular bar code appeared in the sequencing run provided a metric of the relative presence of the corresponding strain in the culture at the time of sampling. Those strains that were present in a culture at the start of the experiment but were absent or depleted by >10 -fold after 9 d of incubation were recorded as defective in survival under that particular starvation condition.

With one significant exception noted below, the two screens yielded nearly identical results. We initially recovered 173 mutants from the plate screen and 238 mutants from the liquid screen that failed to survive glucose starvation, with 102 mutants in common between the two screens. However, only 105 of the 173 mutants recovered in the plate screen were actually identifiably present at time 0 in the liquid screen, so that the liquid screen confirmed 102 of 105 (97%) of the mutants recovered from the plate screen. Similarly, re-examination of the plates for the behavior of those deletion strains identified in the liquid screen revealed that most ($>90\%$) of the mutants identified in the liquid screen survived less well than wild type. We confirmed the correspondence between the plate and liquid screens by retesting—by starvation for glucose in liquid and on plates—24 randomly selected mutants initially identified in one or the other screen. Combining the results of both screens, we identified 299 mutants that were defective in surviving glucose starvation (Supplemental Table S5). In a similar analysis of the results from the nitrogen starvation screens, 70% of the mutants identified in the plate screen were confirmed by the sequence screen, and $>90\%$ of the mutants identified by the sequence screen were initially or subsequently confirmed by the plate screen. Combining the results of the two screens yielded 151 mutants defective in survival during starvation for nitrogen (Supplemental Table S5).

The phosphate screens yielded an unexpected discordance in the results from the two protocols. We recovered 44 mutants from the plate screen and 216 mutants from the liquid screen that were defective in survival for phosphate starvation, with 16 mutants in common (Supplemental Table S5). We were able to confirm 16 of 20 (80%) of the mutants identified in the plate screen were defective in the liquid screen. However, re-examination of the behavior on plates of mutants uniquely identified in the liquid screen failed to reveal any defects in the survival on plates of those deletions relative to wild type. Moreover, retesting several dozen such mutants confirmed that they failed to survive during phosphate starvation in individual liquid cultures but retained normal viability following starvation for phosphate on plates. Thus, unlike the situation with glucose or nitrogen starvation, we could identify two classes of mutants sensitive to starvation for phosphate: One class is defective

whether in liquid cultures or on plates, and the second class is defective for starvation only in liquid.

Analysis of the functions of the genes required for survival under starvation for different nutrients reveals nonoverlapping survival programs. Of the ~ 300 genes whose deletion renders cells sensitive to glucose starvation, more than a third are involved in mitochondrion organization (95 genes; $P = 9.6 \times 10^{-58}$) and cellular respiration (33 genes; $P = 4 \times 10^{-19}$). This is consistent with previous observations that petite mutants lose viability during stationary phase (Martinez et al. 2004). No other significant processes are shared by the remaining genes required for survival during glucose starvation, although deletion of either *SNF1* and *IRA2*, which participate in two of the major glucose-dependent signaling pathways (Zaman et al. 2009), results in sensitivity to glucose starvation. As noted above, we identified 151 genes required for survival following starvation for nitrogen. While a statistically significant number of those overlap with those required for survival following glucose starvation (41 genes), those genes do not define a distinct functional group. Rather, the nonoverlapping genes needed for survival during nitrogen starvation are enriched in those involved in autophagy (21 genes; $P = 9.1 \times 10^{-14}$), particularly microautophagy, and vacuolar transport (25 genes; $P = 3.6 \times 10^{-15}$). Moreover, deletion of any of the nonessential genes encoding products required for signaling through the TORC1 pathway—*TOR1*, *TCO89*, and *RIM15*—are highly sensitive to nitrogen, but not glucose, starvation. Thus, survival during glucose starvation requires mitochondrial function, aerobic respiration, and intact glucose signaling pathways, while survival during nitrogen starvation requires autophagy and signaling through the TORC1 pathway.

The genetic requirements for survival during phosphate starvation overlap those for glucose starvation and nitrogen starvation. Genes required for survival under starvation for phosphate on plates substantially overlap those required for survival under nitrogen starvation; namely, those involved in autophagy, although not those in the TORC1 pathway. Genes required for survival under starvation for phosphate in liquid include also those required for survival for glucose starvation; namely, those involved in mitochondrion organization and aerobic respiration, although not *SNF1*. In sum, these genetic screens reveal two discrete genetic survival programs: respiration and mitochondrial function for survival during glucose starvation, and autophagy and vacuolar function during nitrogen starvation, with phosphate starvation showing some characteristics of each, depending on the details of the environmental conditions. Moreover, the screens indicate that different nutrient signaling pathways participate in the cell's response to different nutritional deprivations.

We compared the genes that are required for survival on starvation for a particular nutrient with those that are induced under that starvation condition and found essentially no overlap. Only four of the >200 genes induced during quiescence under glucose starvation are required for surviving glucose starvation. Similarly, only two of the >150 genes induced during phosphate starvation are required for surviving phosphate starvation. Only two of

the ~200 genes induced under nitrogen starvation are required for surviving that condition. In fact, more genes required for survival upon starvation for a particular nutrient are repressed during that starvation than are induced: Twenty-six out of 356, eight out of 211, and five out of 132 genes transcriptionally repressed under carbon, nitrogen, and phosphate starvation were required for survival under the same condition. Thus, while yeast cells mount both a nutrient-specific transcriptional response and a general starvation response, the genes they induce are not the ones that they need in order to survive the starvation.

Discussion

Quiescent traits emerge during slow growth

Previous work examining quiescent cells obtained by growth into stationary phase identified a number of properties that have come to define quiescence. These properties include heat-shock resistance, thickened cell walls, compacted chromatin, altered transcription patterns, severely attenuated translation, etc. (Gray et al. 2004). However, these studies were based on the comparison of stationary-phase cells with cells growing exponentially in rich medium. Recent work from Lu et al. (2009) and the work presented here indicate that many of the properties ascribed to quiescent cells are observed in cells growing slowly. Namely, heat-shock resistance, resistance to oxidative agents, and thickened cell walls are all properties of quiescent cells that are a direct extension of those attained during slow growth. Similarly, as noted in the present study, many of the transcriptional changes associated with starvation are simply extensions of the pattern exhibited in slow-growing cells. One interpretation of this result is that slow-growing cells actually exit the growth cycle and enter a G_0 state in inverse proportion to the growth rate of the cell. We disfavor this interpretation, since we see very little overlap between mutants that fail to survive stationary phase and those that fail to adapt to slow growth due to limited nutrient addition (M Klosinska and JR Broach, unpubl.). Another possibility is that most of the properties attributed to quiescent cells are manifest in the G_1 stage of the cell cycle, such that slow-growing cells, which spend more time in G_1 than do exponentially growing cells, exhibit quiescent traits in proportion to the amount of time spent in G_1 . However, Elliott and Futcher (1993) showed that all slow-growing cells were stress-resistant regardless of the stage of the cell cycle. Moreover, cells arrested at different stages of the cell cycle are capable of acquiring stress resistance (Elliott and Futcher 1993; Wei et al. 1993). Accordingly, we favor a model in which many of the properties associated with stationary phase, including stress resistance and cell wall integrity, are simply extensions of attributes that manifest in inverse proportion to nutrient availability. This is similar to the expression of growth-regulated genes, whose levels of expression are directly regulated by nutrient signaling pathways in response to nutrient levels (Airolidi et al. 2009). Determining

exactly how these properties are regulated by nutrients requires further analysis. We do note that certain properties, such as expression of a specific subset of genes, do appear to be exclusively a response to entry into quiescence. The work presented here provides a useful identification of those quiescent-specific genes, although their function in quiescence remains unclear.

Do yeast cells access a single quiescent state?

We found that yeast cells subjected to starvation for different nutrients display an acute transcriptional and metabolic response, which, by 24 h, settles into a stable pattern that persists for an extended period of starvation. This transition correlates with cessation of cell cycle progression and acquisition of resistance to various other stresses, such as heat shock or oxidative damage. Under any of the starvation conditions, cells retain significant viability for extended periods of time. Thus, cells exhibit stable behavior after a day of starvation, regardless of the mode of starvation, and acquire properties conferring resistance to multiple stressful conditions. Moreover, the transcription pattern that yeast cells exhibit on starvation is similar across all starvation conditions and resembles the pattern that cells exhibit as an acute response to a variety of stresses—a pattern known as the environmental stress response. However, much of the transcriptional response we observe under starvation—and much of the environmental stress response—is a direct response to cessation of growth and does not represent a program associated specifically with a quiescent state. Nonetheless, after factoring out those growth-dependent transcriptional changes, we can identify a number of genes whose transcript levels change solely as a consequence of starvation, regardless of which nutrient is eliminated. Thus, certain properties appear to define a discrete, albeit limited, quiescent program that is independent of the mode of entry into quiescence.

In contrast to the physiological and transcriptional programs that underlie all starvation responses, the metabolic changes attendant upon starvations and the genetic requirements for surviving starvation are dependent on the particular starvation conditions. Our observations suggest that cells attain a stable metabolic profile upon prolonged starvation that depends specifically on the limiting nutrient and is completely independent of any transcriptional changes that occur in response to that starvation. Moreover, different genes are required for surviving different starvations. Genes required for surviving glucose starvation are highly enriched for those involved in mitochondrial organization and those needed for oxidative phosphorylation. While that set is similar to the genes required for survival in stationary phase, in which cells first deplete glucose and then the resulting ethanol, our protocol does not necessitate that cells metabolize ethanol prior to entry in quiescence. Thus, the requirement for oxidative phosphorylation to survive glucose starvation likely reflects the need to metabolize catabolic products of internal macromolecules during starvation. In contrast, the genes required to survive

nitrogen starvation are enriched for vacuolar transport and autophagy, suggesting that cells maintain viability under this condition by reallocating limited nitrogen through autophagic degradation of existing proteins and organelles. Thus, the genetic requirements for surviving starvation differ depending on the starvation conditions, suggesting that quiescent cells have different needs dictated by the event inducing quiescence.

We note that intact nutrient signaling pathways are required for survival during starvation for the cognate nutrient. Tco89, Tor1, and Rim15 are required for surviving nitrogen starvation, although not glucose or phosphate starvation. The first two proteins are components of the TORC1 complex, whose activity is correlated with high amino acid levels and an adequate nitrogen source, and Rim15 is a downstream target of TORC1 previously identified as being required for entry into quiescence and meiosis (Vidan and Mitchell 1997; Cameroni et al. 2004). While Rim15 would be expected to emerge from our screen, Tco89 and Tor1 would not. In fact, Boer et al. (2008) demonstrated that mutations that reduce TORC1 signaling protected leucine auxotrophs from leucine starvation. We do not fully understand these results, but they may indicate that responsiveness of cells to nutrient levels, not just a reduction in signaling, may be important for the complex process of entry, maintenance, and exit from quiescence. We also observed that deletion of *SNF1* or *IRA2*, which encode components of the glucose-responsive AMP kinase and the Ras/protein kinase A (PKA) pathways, precludes successful survival upon glucose starvation, but not upon phosphate or nitrogen starvation. Both of these deletions result in elevated glucose signaling, resulting in the cell's misperception that glucose is present even when it is not (Zaman et al. 2008; Smets et al. 2010). Accordingly, we infer that the failure of these mutants to survive glucose starvation results from their inability to perceive glucose starvation or mount an appropriate response to that condition. *IRA2* is the only nonessential, nonredundant gene encoding a component of the Ras/PKA pathway, and so it is the only gene present in the deletion collection that would yield elevated PKA signaling. However, deletion of *BCY1* or introduction of an activated allele of *RAS2*, either of which results in a significantly higher level of PKA activity in the cell than does deletion of *IRA2*, has been shown previously to render cells sensitive to carbon starvation as well as nitrogen starvation—an observation that we confirmed with our strains. In sum, although extreme activity of nutrient signaling pathways generally precludes survival in any quiescent state, we conclude from our data that survival on different starvations requires different nutrient signaling pathways.

We found that the genes required for surviving a particular starvation condition do not overlap those that are induced under that starvation condition. This observation does not take into account those genes that are functionally redundant, such as *MSN2* and *MSN4*. However, the list of genes induced in quiescence does not include many with functionally redundant paralogs, suggesting that redundancy does not account for the discrepancy in transcriptional induction and genetic require-

ments in quiescence. Our results are consistent with those obtained recently for survival of acute heat shock, which documented that the genes induced by heat shock are not the same as those required for surviving heat shock (C Lu and D Botstein, pers. comm.). These findings are also reinforced by a study in which genes identified as up-regulated during anaerobic growth were deleted; the resulting deletion strains, for the most part, did not have detectable growth defects under anaerobic conditions (Tai et al. 2007). One explanation for these observations is that cells use one stress as an indicator that other stresses are likely to follow, and thus mount a transcriptional response to deal with a broad collection of anticipated insults (Tagkopoulos et al. 2008). However, the lack of any correlation between genes induced by starvation and those required to survive starvation argues that cells do not rely on transcriptional induction as a means of coping with the stress in which they find themselves, perhaps because the resultant lag in response due to the time required for transcription and translation, compounded by the paucity of resources to fuel those changes, would render the response too late. Rather, we anticipate that more immediate adaptations, such as post-translational modifications, may underlie the acute response of cells to immediate insults.

In sum, the results presented in this study suggest that yeast cells do not enter a singular state as a means of maintaining viability in response to prolonged deprivation of nutrients. Rather, the data are consistent with the interpretation that cells can access at least two, and perhaps more, distinct states when confronted with nutrient deprivation, and that cells respond to such stresses in a manner calculated to maximize survival under the specific condition in which it finds itself. This observation is consistent with findings with cultured mammalian cells that suggest that the program elaborated by a cell to promote survival is highly dependent on the growth inhibitory condition, such that quiescence attained by growth factor limitation is distinct from that attained by contact inhibition (Coller et al. 2006). Certainly, the yeast system offers an opportunity to define the genetic distinctions among different quiescent conditions and provide insights into the distinctions among different quiescent states.

Materials and methods

Strains, media, and culture conditions

Metabolite and transcript analyses were performed using the prototrophic *MATa* W303 derivative formerly designated DBY15001 and renamed Y3358 for this study. Supplementary microarray studies were conducted with a *MATa* CEN.PK-derived prototroph formerly designated DBY10085 and renamed Y3840 (van Dijken et al. 2000). SD consisted of 6.7 g/L yeast nitrogen base without amino acids (Difco) and 20 g/L glucose. Limiting and starvation media were based on SD and are explicitly described in Supplemental Table S1. All solid starvation media used triple-washed agarose rather than agar as a solid support. The prototrophic deletion collection was created by Drs. Amy Caudy and David Hess from the existing deletion collection from Research Genetics by backcrossing to CEN.PK using Synthetic Genetic Array techniques.

Chemostat cultures were prepared and grown as described previously (Saldanha et al. 2004; Brauer et al. 2008). An overnight

culture grown in limited medium was used to inoculate a 300-mL working volume chemostat containing glucose-limited, nitrogen-limited, or phosphate-limited media (Supplemental Table S1). The chemostat was grown in batch mode for ~12 h, then switched to continuous mode with a dilution rate ranging from 0.05 h⁻¹ to 0.32 h⁻¹. Chemostat cultures were grown at 30°C to steady state (~3–5 d) and then sampled. Starved batch cultures were prepared by first growing cells to early exponential phase in glucose-limited, nitrogen-limited, or phosphate-limited chemostat media; harvesting cells by centrifugation; resuspending them in respective starvation media; and incubating them for 4 d at 30°C.

Metabolite and transcript analyses

Cells for transcript and metabolite analysis were collected and treated essentially as described previously (Brauer et al. 2006). Briefly, cells from three to four single colonies were grown in liquid SD, nitrogen-limited, or phosphate-limited media to an OD₆₀₀ of 0.1 and then filtered onto nylon filters (GE, Magna nylon 0.45-μm, 47-mm diameter filters). Filter membranes were incubated on agarose plates with respective limited media for 2.5 h and then transferred to starvation plates. For metabolite analysis, samples were collected at specific time points by transfer to -20°C 40/40/20 acetonitrile/methanol/water. Metabolites were extracted using a filter quench protocol (Crutchfield et al. 2010) and maintained at 4°C until analysis by LC-MS/MS (Boer et al. 2010).

For transcript analysis, three to five filters were collected for each starvation per time point and immersed in liquid nitrogen. Total RNA was extracted using the Qiagen RNeasy Mini kit, including the additional DNase I digestion. cRNA for microarray hybridization was synthesized following the standard protocol of the Agilent Low RNA Input Linear Amplification kit (Agilent Technologies). cRNA was extracted using the Qiagen RNeasy Mini kit and hybridized to Agilent Yeast Gene Expression Microarray (version 1, 4x44K, G2519F, or version 2, 8x15K G4813A) slides and scanned at 5-μm resolution. Data were extracted using Agilent Feature Extraction software version 9.5 with Linear Lowess dye normalization and no background subtraction and were submitted to the Princeton University Microarray database for storage and analysis. Normalized data were filtered to exclude features flagged with low intensity and to include only features with both red and green intensities well above background. Finally, of the genes that passed the spot filter criteria, only genes with >80% good data across all experiments were included in subsequent analyses. All microarray data described in this report can be downloaded from http://puma.princeton.edu/cgi-bin/publication/viewPublication.pl?pub_no=534, and additional supporting material can be found at <http://genomics-pubs.princeton.edu/quiescence>.

To compare the nutrient starvation data collected in this study to the chemostat limitation data presented in Brauer et al. (2008), we created common reference files. The nutrient starvation arrays used day 0 of the starvation as the reference channel, whereas the Brauer et al. (2008) data set used a reference collected under chemostat cultivation in carbon limitation at a growth rate of 0.25 h⁻¹. Therefore, we ran three further arrays, one per elemental nutrient, to correct for the differences between these references. In these arrays, the reference channel was RNA collected under the same conditions as the Brauer et al. (2008) reference, and the experimental channel contained RNA that had been used as the reference from the nutrient starvation experiments. The expression values from these arrays were added to the quiescent expression data, yielding data about the quiescent state that was directly comparable with the Brauer et al. (2008) data set. To avoid any potential bias introduced by saturation of fluorophores, we

excluded genes that had any probes flagged as saturated in the Brauer et al. (2008) limitation data, the common reference arrays, or the starvation data.

To identify genes regulated by growth rate and by quiescence, each gene was fit to a linear model that included (1) a growth-rate slope (assuming a growth rate of 0 for quiescent time points); (2) nutrient-specific effects for carbon, nitrogen, and phosphorus; and (3) a quiescence-specific effect:

$$\log_2(E/E_g) = mG + cC + nN + pP + qQ + \varepsilon.$$

Here, E is the gene expression level of a gene; E_g is the geometric mean expression level of that gene (i.e., arithmetic mean expression level in log space); and m , c , n , p , and q are model parameters. G is a vector corresponding to the growth rate, varying from 0.3 h⁻¹ to 0.05 h⁻¹ in limitation and set to 0 during nutrient starvation; C , N , and P are vectors equal to 1 in carbon, nitrogen, or phosphate limitation, respectively, and equal to 0 everywhere else; Q is a vector that is equal to 1 in quiescence and equal to 0 everywhere else; and ε is the residual (i.e., uncaptured variation) from the model fit. Model parameters were tested for a significant difference from 0; the resulting P -values were then corrected using the Benjamini-Yekutieli FDR (Benjamini and Yekutieli 2001). From this analysis, we classified genes as exhibiting quiescence-specific expression when they have value of both $|q| > 1$ (i.e., twofold induced or repressed) at an FDR of 5% and m not significant for all starvation conditions. Similarly, we classified genes as exhibiting growth rate-dependent expression when they have both a nonzero value of m , significant at a 5% FDR, and $|q| < 0.32$ (i.e., <1.25-fold). Genes with significantly nonzero values of both q and m were classified as having both growth rate-specific and quiescence-specific expression. The above analysis was repeated for both the CEN.PK and W303 data; only genes that were identified in both strains as quiescence-specific, growth rate-specific or both were assigned these labels. Finally, we could also identify genes that had quiescent-specific and/or growth rate-specific expression for only one or two starvation conditions, but not for the other(s), by fitting a version of the model including an interaction effect between Q and N . Genes identified as having a quiescence-specific expression pattern under a given nutrient were required to have an effect size of at least twofold and be present at an FDR of 5%, and were required to meet these criteria using the data from each strain, as above.

The growth rate and quiescence regressors G and Q are, intrinsically, negatively correlated (Pearson's $r = -0.76$; 95% CI: $-0.89 < r < -0.54$). Since collinearity can lead to inaccurate parameter estimation in standard multivariate regression, we also estimated the above parameters using regularized ridge regression. Ridge regression requires a tuning constant λ , which was determined by generalized cross-validation. The correlation between growth rate-specific and quiescence-specific effects was reduced ($P = 1.4 \times 10^{-3}$, Pearson's $r = 0.048$; 95% CI: $0.018 < r < 0.077$). The growth rate and quiescence coefficients obtained from ridge regression were nearly perfectly matched with those obtained via standard multivariate regression (growth rate: Pearson's $r = 0.99$, Lin's $r_c = 0.986$; quiescence: Pearson's $r = 0.99$, Lin's $r_c = 0.987$), indicating that the parameter estimation was largely stable with respect to the correlation in the predictors. We performed these analyses in the R statistical software environment (<http://www.r-project.org>) with the MASS software package (Venables and Ripley 2002).

Quiescence viability screens

To identify mutants that were unable to survive starvation on plates, we pinned the prototrophic deletion collection onto SD plates (Singer PlusPlates) using Biomek FX Laboratory Automation

Workstation and, after 2 d of incubation at 30°C, transferred it to another set of SD plates using the Singer RoToR HAD robot to ensure uniformity. After incubation for 1 d, the collection was pinned onto SD, nitrogen-limited, and phosphate-limited sets of plates using fresh RePads for each set and the Singer robot. After 1 d of growth at 30°C, the collection was transferred to glucose, nitrogen, or phosphate starvation plates, respectively. The plates were incubated for 9 d at 30°C in a humidity chamber. The colonies were then transferred to YEPD plates, grown for 2 d at 30°C, and scanned, and the data were analyzed. Those strains that failed to form a colony on the YEPD plate but that had been present on the prestarvation plate were scored as defective in surviving that starvation condition.

To identify mutants that were unable to survive starvation in liquid, we inoculated a sample of the pooled prototrophic deletion collection into 500 mL of SD, nitrogen-limited, or phosphate-limited media. They were grown until reaching OD (at 600 nm) of ~0.5, then an aliquot was taken and the rest were spun down and resuspended in glucose, nitrogen, or phosphate starvation media. The aliquot was used to plate ~2 million cells onto YEPD plates (15-cm diameter), 30,000 per plate. After 9 d of starvation, another aliquot was taken from the liquid cultures and likewise plated. To compensate for the loss of viability during starvation, and to get a colony density similar to that obtained for cells from the exponentially growing culture, 90,000 cells were plated per plate. In both cases, plates were incubated ~40 h at 30°C, and colonies were scraped off in 1 mL of water per plate, pooled (each condition separately), mixed thoroughly, and frozen at –80°C. DNA was extracted from 1 mL of each pellet using a standard protocol with ethanol precipitation, standard PCR was run to amplify the UP tag and DOWN tag of each mutant, PCR product was run on an agarose gel, and gel was extracted using the Qiagen Gel Extraction kit. Afterward, the DNA concentration was measured using the Quant-iT Assay (Invitrogen) using the Qubit fluorometer (Invitrogen), and the samples were sequenced using the Solexa sequencer. Sequences were assigned to ORFs and quantified.

In collating the results of these screens, we first identified those genes in the sequence screen that were present at significant levels at day 0 and were absent or depleted by >10-fold at 9 d of starvation. We also identified those genes that failed to grow after starvation in the plate-based screen. Those genes common to both of these lists were retained as confirmed mutants. Of those genes unique to the plate screen, we determined whether they had been present at day 0 in the sequence screen. Those that were not were added to the confirmed mutants. We then culled from the final list any dubious ORF that scored as mutant and was adjacent to or overlapped a confirmed gene that was identified as mutant in the screen.

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Yeast cells can access distinct quiescent states

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