System-Level Analysis of Genes and Functions Affecting Survival During Nutrient Starvation in *Saccharomyces cerevisiae*

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ABSTRACT

An essential property of all cells is the ability to exit from active cell division and persist in a quiescent state. For single-celled microbes this primarily occurs in response to nutrient deprivation. We studied the genetic requirements for survival of *Saccharomyces cerevisiae* when starved for either of two nutrients: phosphate or leucine. We measured the survival of nearly all non-essential haploid null yeast mutants in mixed populations using a quantitative sequencing method that estimates the abundance of each mutant on the basis of frequency of unique molecular barcodes. Starvation for phosphate results in a population half-life of 337 hours whereas starvation for leucine results in a half-life of 27.7 hours. To measure survival of individual mutants in each population we developed a statistical framework that accounts for the multiple sources of experimental variation. From the identities of the genes in which mutations strongly affect survival, we identify genetic evidence for several cellular processes affecting survival during nutrient starvation, including autophagy, chromatin remodeling, mRNA processing and cytoskeleton function. In addition, we found evidence that mitochondrial and peroxisome function are required for survival. Our experimental and analytical methods represent an efficient and quantitative approach to characterizing genetic functions and networks with unprecedented resolution and identified genotype by environment interactions that have important implications for interpretation of studies of aging and quiescence in yeast.
INTRODUCTION

The normal life of most cells comprises alternating periods of growth and quiescence. Commitment to the mitotic cell cycle represents a critical decision for a cell and requires mechanisms for determining that internal and external conditions are sufficient to ensure successful traversal of the cell division cycle. The application of genetic approaches to the study of the cell division cycle in the model organisms *Saccharomyces cerevisiae* (budding yeast) and *Schizosaccharomyces pombe* (fission yeast) yielded a detailed understanding of the molecular processes underlying progression of the cell division cycle (HARTWELL et al. 1974; JOHNSTON et al. 1977; NURSE 1975). Subsequent work showed that the major molecules and principles of cell division are conserved from yeast to humans and that dysregulation of the cell cycle is a hallmark of cancers (HARTWELL 1991; HARTWELL 2002; NURSE et al. 1998; NURSE 2002). By contrast with our understanding of the mitotic cell cycle, our knowledge of the molecular processes that govern exit from the cell cycle and prolonged maintenance of a viable non-proliferative cellular state in both humans and model systems is poor. A detailed understanding of the molecular mechanisms mediating cell cycle exit and maintenance of a viable quiescent state is critical to understanding normally functioning cells, which are primarily post-mitotic, and their aberrant states.

Yeast cells cease active cell division when external conditions are unfavorable for continued growth (JOHNSTON et al. 1977; LILLIE and PRINGLE 1980). The extracellular status of compounds containing essential elements such as carbon, sulfur, nitrogen and phosphorus is particularly important and when these elements are in low abundance cells exit the cell cycle. Specialized sensing mechanisms appear to uniquely determine the status of different essential nutrients and connect to signaling pathways such as the
protein kinase A, RAS and TOR pathways that mediate myriad downstream effects impacting the transcriptional, translational and metabolic state of the cell (ZAMAN et al. 2008). Remarkably, the different environmental signals result in the identical phenotypic outcome: arrest in the G0/G1 stage of the cell division cycle. In yeast, exit from the cell cycle is associated with a number of physiological characteristics including increases in the carbohydrates trehalose and glycogen, a thickened cell wall, condensation of chromosomes, increased vacuolar volume and increased resistance to stress (GRAY et al. 2004; WERNER-WASHBURNE et al. 1993). Global studies have shown that cell cycle arrest associated with nutrient starvation is associated with dramatic alterations in transcript (BRAUER et al. 2005; GASCH et al. 2000; SALDANHA et al. 2004) and metabolite (BRAUER et al. 2006) levels. Many of these changes appear to be consistent with an extrapolation of responses in transcript (BRAUER et al. 2008) and metabolite (BOER et al. 2010) levels associated with decreasing growth rates, as determined in steady-state chemostat cultures. However, it remains unclear whether prolonged starvation results in a distinct cellular state (i.e. a G0 state) or whether cell cycle arrest is essentially identical to G1 of the cell division cycle. Furthermore, it is unclear whether quiescent states induced by deprivation of different nutrients are equivalent.

Starvation for nutrients such as carbon, phosphate, nitrogen and sulfur results in a uniform cell cycle arrest of the population, which is readily detected by the near complete absence of budded cells in the culture (LILLIE and PRINGLE 1980). We have previously referred to these nutrient conditions as “natural limitations” in contrast to “unnatural limitations”, which can be imposed by starving an auxotrophic strain for its auxotrophic requirement (SALDANHA et al. 2004). Importantly, in the case of unnatural limitations individual cells do not uniformly arrest as
unbudded cells upon cessation of culture growth (Saldanha et al. 2004). Populations of prototrophic strains starved subjected to natural limitations maintain high viability for periods greater than 100 days (Lillie and Pringle 1980). In contrast, starvation of an auxotrophic strain for its auxotrophic requirement results in dramatically reduced viability (Boer et al. 2008). Using a genetic selection, we previously showed that the poor survival of auxotrophs can be suppressed by loss of function mutations in TOR1, SCH9, a component of the PKA pathway and PPM1, a regulator of protein phosphatase 2A function (Boer et al. 2008).

The survival of yeast cells during prolonged periods of starvation has also been used as a model of chronological aging of post-mitotic cells (Fabrizio and Longo 2003). In studies that aim to use yeast as a model for this fundamental process the PKA and TOR pathways have been implicated in post-mitotic aging in yeast (Burtner et al. 2009; Fabrizio et al. 2001; Powers et al. 2006; Steffen et al. 2008) and it has been argued that these same pathways underlie aging phenotypes in humans (Longo 2003). Although several of the loci identified in our original genetic screen (Boer et al. 2008) overlap with those identified in chronological aging studies (Fabrizio et al. 2001; Kaehlerlein et al. 2005b; Powers et al. 2006), it has remained unclear whether mutations in these same components influence the survival of strains starved for naturally limiting nutrients such as sources of carbon, sulfur, nitrogen and phosphorus.

To identify the genetic factors required for cell cycle exit and survival during prolonged nutrient starvation we performed complete screens of the 4,811 haploid gene deletion mutants (Giaever et al. 2002) starved for two different nutrients. We performed this screen under conditions of phosphate starvation, a natural nutrient limitation, and leucine starvation, an unnatural nutrient limitation. Because our survival assay requires repeated measurements of viability over the period of starvation
(Boer et al. 2008), the analysis of individual mutants is prohibitively time consuming and expensive. Therefore, we made use of the fact that each mutant is tagged by two unique molecular barcodes of 20 base pair length that flank the antibiotic resistance cassette and can be amplified using common PCR priming sites (Giaever et al. 2002). Previously, multiplexed analysis of mutants has been performed using DNA microarrays to assay the relative abundance of molecular barcodes as a means of estimating the abundance of each mutant (Giaever et al. 2002; Ho et al. 2009; Pierce et al. 2007; Yan et al. 2008). More recently, this approach has been adapted to high throughput sequence analysis (Smith et al. 2009). We independently developed a quantitative method using high throughput sequencing of barcodes similar to (Smith et al. 2009) for measuring the abundance of individual mutants in heterogeneous pools of mutants. We established a statistical framework for analyzing data obtained from this quantitative barcode sequencing of complex mixtures of mutants over time that accounts for the multiple sources of variation in these experiments.

Through application of our quantitative barcode sequencing method we quantified the survival of individual mutants when starved for either phosphate or leucine over a three-week period. Using the increased resolution of quantitative sequencing of barcodes and multiple sampling during the starvation period we were able to determine individual survival profiles in each condition for more than half the genes in the genome. Using an exponential death model estimated by Poisson generalized linear regression we determined the rate of death for each mutant in both conditions, allowing us to treat survival as a quantitative trait.

Through bioinformatic analysis of the genes affecting survival, we found that cell cycle exit, aging and survival are multifactorial processes involving several molecular functions. We found that mitochondrial function is critical to survival of cells starved for phosphate and
enhances the rapid loss of viability observed in leucine starved cells, indicating that respirative metabolism is required for proper response to nutrient depletion even in the presence of excess glucose. Our method identified genetic evidence for important roles of autophagy in the survival of cells starved for phosphate and leucine. We also find evidence for several additional processes and functions including mRNA processing, chromatin remodeling and cytoskeleton functions that appear to be uniquely related to survival in phosphate starvation conditions.

Our experimental and analytical methods represent a general approach to quantitative multiplexed genetic screens that make possible new types of genetic studies in which the aim is to identify those genetic modules that function in particular cellular processes and events. By treating heretofore-qualitative phenotypes as quantitative traits it may be possible to achieve a higher resolution genotype-phenotype map and infer new relationships between genes and the processes that they govern.

**MATERIALS AND METHODS**

**Strains:** The *Saccharomyces cerevisiae* haploid deletion collection (YSC1063, Open Biosystems, YKO *Mata his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*) was manually transferred as clonal isolates from 96 well glycerol stock plates to YPD agar plates + 200μg/mL G418 and grown at 30°C for 3 days. To construct the mixtures of all mutants, complete colonies were harvested by addition of 5mL of water to each plate and subsequently pooled. Glycerol was added to a final concentration of 15% and aliquots of 2 mL, containing 1.8 x 10⁹ cells/mL, were frozen at -80°C.

**Media and growth conditions:** Chemically defined media were based on (SALDANHA et al. 2004), with modifications to chemically complement auxotrophies present in the deletion collection strain. For both phosphate and leucine limiting media we used 5.0g/L (NH₄)₂SO₄, 0.50g/L MgSO₄·7H₂O,
0.10g/L CaCl₂.2H₂O, 0.10g/L NaCl, 40mg/L histidine, 40mg/L uracil, 60mg/L lysine. For phosphate limiting media we added 200mg/L leucine and 1.0g/L KCl. The only source of phosphorus in phosphate limiting media was KH₂PO₄, which was present at an initial concentration of 5mg/L. For leucine limiting media we added 1.0g/L KH₂PO₄ and 20mg/L leucine. We made each medium both with and without 200μg/mL G418. Phosphate and leucine concentrations are well within previously defined limiting ranges (BOER et al. 2008; SALDANHA et al. 2004).

We inoculated 400mL of medium with 1.6 mL (2.9 x 10⁹ cells) of the pooled deletion collection pool in 500mL vessels (Infors). The culture was grown at 30°C, agitated at 400rpm with an impeller and aerated with 5 L/min filtered and humidified air. The pH was constantly measured and maintained at 5.0 throughout by the automated addition of 0.1M KOH. Samples of 1mL volume were taken at 0, 24, 48, 72, 99, 144, 240 and 473 hours.

**Determination of population parameters:** Each sample was sonicated to disrupt adhesive cells. The number of cells/mL and the average cell volume were determined using a Beckman Coulter counter. The number of viable cells was determined by manually counting colony-forming units (CFUs) after plating a known number of cells on YPD-agar plates and incubating at 30°C for 2 days. Each data point is an average of at least 2 replicates. Typically, 1000 colonies were counted per time point when viability permitted. Population viability was defined as the percentage of plated cells that formed a CFU.

**Extraction of DNA from viable fraction of mutant populations:** To enrich for viable cells we performed an overnight outgrowth of the starved population. We placed a 1mL sample of the starved population in 50mL of supplemented minimal medium (5.0g/L (NH₄)₂SO₄, 1.0g/L KH₂PO₄, 0.50g/L MgSO₄.7H₂O, 0.10g/L CaCl₂.2H₂O, 0.10g/L NaCl, 40mg/L histidine, 40mg/L
uracil, 60mg/L lysine, 200mg/L leucine, trace metals and vitamins and 2% glucose). Cells were grown for 24 hours in supplemented minimal medium before being harvested for DNA isolation. Genomic DNA was extracted using the Qiagen Genomic Tip system.

**PCR amplification for quantitative barcode sequencing:** We amplified molecular barcodes from genomic DNA and incorporated adaptors for the Illumina Genome Analyzer in a single step using tailed PCR primers (Illumina Genome Analyzer adaptor sequences are underlined in italics). Uptags were amplified using the primers Illumina-uptag (5’-AAT GAT ACG GCG ACC ACC GAG ATC T GAT GTC CAC GAG GTC TCT- 3’) and Illumina-UPKANMX (5’ – CAA GCA GAA GAC GGC ATA CGA GTC GAC CTG CAG CGT ACG -3’). Downtags were amplified in a separate reaction with the primers Illumina-downtag (5’ AAT GAT ACG GCG ACC ACC GAG ATC T CGG TGT CGG TCT CGT AG- 3’) and Illumina-DNKANMX (5’ CAA GCA GAA GAC GGC ATA CGA GAA AAC GAG CTC GAA TTC ATC G- 3’). We amplified barcodes using a high fidelity thermostable polymerase (TaKaRa PrimeSTAR) from 200ng of genomic DNA in 20μL reactions with each primer at a final concentration of 1μM. PCR products were visualized on a 2% agarose gel to confirm the generation of a ~110bp PCR product. We purified PCR products from excess primer and PCR reagents using Qiagen PCR cleanup columns. We quantified the total yield of purified PCR product using a fluorometer and diluted the ~110bp product to 10nM concentration corresponding to concentration of 0.68ng/μL.

**Quantitative barcode sequencing using an Illumina Genome Analyzer:** We combined the PCR products from the separately amplified uptag and downtag reactions from each sample in equimolar amounts and added them to the same lane of an Illumina Genome Analyzer flow cell following the manufacturer’s protocol. To simultaneously sequence the uptags and downtags in the same lane we added two custom sequencing primers in equimolar amounts that were complementary to either the uptag or downtag sequence. The uptag-
Sequencing primer (5'- CGA CCA CC GAGA TCT GAT GTC CAC -3') is complementary to the U1 sequence and the downtag-Sequencing primer (5'- GAC CAC CGA GAT CTC GGT GTC GGT C -3') is complementary to the D1 sequence of the deletion cassette. Both sequencing primers lie three bases from the start of the barcode sequence. This enables sequencing of a 3 nucleotide sequence that identifies the barcode as either an uptag (sequence index TCT) or a downtag (sequence index TAG) before sequencing the unique 20 base barcode sequence.

Barcodes were sequenced using 36 base sequencing cycles. Sequencing data were processed using the Illumina pipeline (Gerald, Bustard). Fastq files for each sequencing run were processed with custom Perl scripts to determine the identity and number of each barcode. A complete list of sequencing experiments and number of mapped barcode reads is provided (Table S1).

Normalization of barcode counts: We determined the total number of uptags and downtags sequenced in each lane using the unique three base prefix. We determined the total number of counts for each unique known barcode. We tested only for exact matches to known barcodes and did not consider individual base quality metrics. For each sequence run, approximately 25% of barcodes could not be exactly mapped to a known barcode. Therefore, the presence of the three base index was essential for accurate normalization of uptag and downtag counts. For initial comparisons and cluster analyses we converted barcode counts to a frequency by dividing the abundance of each unique barcode by the total number of uptags or downtags.

For modeling the death rates of strains over the time course, we normalized the data within each starvation experiment to preserve the scale of the original barcode counts. Briefly, let \( x_i(t_j) \) be the number of reads for barcode \( i \) at time point \( t_j \), \( (t_1 = 24, t_2 = 48, \ldots, t_7 = 473) \) and let \( N_j \) be
the total number of reads corresponding to sequencing lane \( j \). We formed weights \( w_j = 7 \times N_j / \sum_{k=1}^{7} N_k \) and calculated normalized counts by \( y_i(t_j) = w_j \times x_i(t_j) \) for each barcode \( i \) and lane \( j \).

**Cluster analysis:** We performed hierarchical cluster analysis using Cluster 3 (DE HOON et al. 2004). For clustering we computed the Euclidean distance between log$_2$-transformed proportional data for each time point that had been normalized to the initial time point \( t=24 \) hours using complete linkage. Clustering was visualized using JavaTreeView (SALDANHA 2004). Gene ontology (GO) term enrichment analysis was performed using GoTermFinder (BOYLE et al. 2004).

**Model fitting and inference:** All model fitting and inference was performed using the statistical software package R. As described above, let \( y_i(t_j) \) be the normalized number of sequencing reads for barcode \( i \) at time point \( t_j \). We initially modeled \( y_i(t_j) \sim \text{Poisson}(\exp[a_i - b_i t_j]) \), where \( b_i \) quantifies the relative (to the total population) death rate per hour for the strain corresponding to barcode \( i \), and \( h_i = \ln(2)/b_i \) represents the relative half-life. We performed a Poisson generalized linear regression to estimate \( a_i \) and \( b_i \). An inspection of the deviances revealed that there is a systematic over-dispersion, meaning the variance is larger than expected under the Poisson model. This makes sense because there are several levels of variation experimentally induced beyond the assumed exponential death rate. Therefore, we fit the above model using an over-dispersed Poisson error model (via the R function glm with family=quasipoisson).

The counts for each barcode at a specific time point measure the abundance of the corresponding strain relative to the entire population. Therefore, a “flat” pattern across time points \( (b_i=0) \) according to the above model indicates the strain corresponding to that barcode dies at a rate equal to the overall population death rate. In order to quantify the
absolute death rates, we took into account the percentage of the population alive at each time point (see Determination of population parameters above). Let \( p_1, p_2, \ldots, p_7 \) be the percentages corresponding to the seven time points at which sampling occurred. We calculated weights 
\[
v_j = \frac{7 \times p_j}{\sum_{k=1}^{7} p_k}
\]
to form counts \( z_i(t_j) = v_j \times y_i(t_j) \) that capture the absolute change in strain abundance over time. We modeled the \( z_i(t_j) \) according to an over-dispersed Poisson generalized linear regression as before to obtain estimates of the absolute death rates and half-lives. In this case a “flat” pattern across time points (\( b_i = 0 \)) indicates that the strain has no death across the time points.

All inference (hypothesis testing and confidence intervals) on the death rates \( b_i \) and half-lives \( h_i = \ln(2)/b_i \) was performed using the default settings from the glm function under the over-dispersed Poisson model (family=quasipoisson) in R. Specifically, p-values were obtained for each barcode \( i \) in testing \( b_i = 0 \) for both the relative and absolute analyses, and false discovery rate (FDR) q-values were obtained to form significance thresholds (STOREY and TIBSHIRANI 2003).

Gene Function enrichment analysis: We performed gene class function enrichment analysis by comparing the distribution of half-lives for defined subsets of genes to the distribution of all half-lives in which the values for the query set had been removed. We assessed the statistical significance of the difference between these two distributions using a Wilcoxon-Mann-Whitney test in R. Gene sets were defined by gene ontology (GO) terms from all three ontologies (process, function, location) and GO Slim terms, biochemical pathways and protein complexes as defined using annotated data available through SGD (http://downloads.yeastgenome.org/literature_curation/) accessed on March 9th 2010. In addition, we used high throughput studies of the yeast deletion collection and a subset of genome-wide transcript profiling
studies to define phenotypic classes. We applied a p-value cutoff of 0.001 for identifying gene classes exhibiting non-random distributions of half-lives. Given that we tested 528 gene classes, this implies we expect 0.5 false positives among the results that pass this significance threshold.

RESULTS

The first nutrient depleted determines the rate of population death:

To investigate the survival of all viable haploid (MATα) deletion mutants to defined starvation states we inoculated cultures with mixtures containing the entire mutant library. To ensure precise control of the starvation conditions we used media that are similar to the commonly used yeast minimal media, but composed entirely of chemically defined components (methods). In these media all components are in excess and one nutrient is present at limiting concentration, i.e. it is the first nutrient that is exhausted in the culture. Allowing cells to deplete the limiting nutrient rather than switching cells to medium lacking a particular nutrient allows cells to adjust to decreasing amounts of the limiting nutrient. This is likely to be important as previous studies suggest that starvation responses are initiated well before nutrients are depleted and cell cycle exit occurs (Lillie and Pringle 1980). The genotype of all deletion mutants contains four auxotrophic alleles: his3Δ1, leu2Δ0, lys2Δ0 and ura3Δ0. To compare survival of mutants in response to a natural and an unnatural limitation we used media in which either phosphate or leucine is the first nutrient exhausted using known limiting concentrations (Boer et al. 2008; Saldanha et al. 2004).

We inoculated four independent cultures with 2.9 x 10^9 cells each. Thus, each of the ~4,800 viable deletions is initially represented by approximately 6 x 10^5 clonal individuals in the inoculums. Each of the two nutrient limiting conditions was imposed in two separate cultures that
differed only by the presence of the antibiotic, kanamycin, to aid in maintaining culture sterility. Following 24 hours of culture growth we measured survival of mutants in each starvation condition by determining population viability at multiple time points. We observed a dramatic and reproducible difference in survival profiles that distinguishes populations starved for leucine from those starved for phosphate (Figure 1A). In the case of phosphate starvation, the viability of the heterogeneous mutant pool remains high throughout the starvation period and approximately 30% of the population is viable following nearly 500 hours of starvation. In contrast, populations starved for leucine survive poorly and less than 1% of the population is viable after starvation for leucine over the same time period. We quantified population survival according to an exponential death rate, and applied a Poisson generalized linear model to the viability data in each experiment. We determined that the mixed population of null mutants starved for phosphate dies at a rate of 0.20%/hour (population half-life = 337 hours) whereas the same population of mutants starved for leucine dies at a rate of 2.5%/hours (population half-life = 27.7 hours). This order of magnitude difference in loss of viability is consistent with our previous results using individual strains (Boer et al. 2008) and demonstrates that the fate of starved cells depends on which nutrient is exhausted in the population first.

Population sizes of approximately $1.5 \times 10^{10}$ and $1.3 \times 10^{10}$ in the phosphate and leucine starved conditions respectively remained essentially constant in replicate populations throughout the starvation periods (Figure 1B). The turbidity of phosphate-starved populations appeared to increase gradually during the starvation period whereas the leucine starved population appeared to decrease (Figure 1C). This may be related to the fact that average cell volume increased in phosphate starved populations but decreased in leucine starved populations (Figure 1D). We have observed
a similar trend in cultures grown under continuous phosphate or leucine limitation in chemostats (Brauer et al. 2008) and this size difference may reflect differences in the volume of vacuoles associated with starvation.

**Quantitative barcode sequencing:** The central challenge in performing multiplex screens of mutant populations is accurate determination of the abundance of each individual mutant. When the yeast gene deletion collection was constructed unique molecular sequences of 20 nucleotides were included on either side of the kanamycin resistance marker used to replace each open reading frame (Giaever et al. 2002). These molecular barcodes, which can be PCR-amplified using common priming sites, enable the identification of each mutant by assaying for the presence or absence of each unique barcode. Typically, mutant identification in pooled experiments has been achieved by hybridization of the PCR products to DNA microarrays containing sequences complementary to each barcode (Giaever et al. 2002). Measurement of the relative abundance of each barcode makes it possible to infer the frequencies of each mutant in a complex mixture of genotypes.

We reasoned that quantitative sequencing of molecular barcodes using new high throughput sequencing methods would provide increased accuracy and dynamic range. Therefore, we developed a method of quantitative barcode sequencing to estimate the frequency of each mutant in heterogeneous populations. This method is designed for use with the Illumina Genome Analyzer 2 and is similar to a recently reported method (Smith et al. 2009). In addition, we developed new methods for analyzing data generated using this approach (see methods).

We undertook a series of control experiments to validate the utility of quantitative sequencing as a means of determining barcode frequencies in mixed populations. First, to determine the false negative rate due to sequencing error, we sequenced a single barcode. We obtained 2,340,984
sequences that passed sequence analysis filters (see Table S1 for a complete summary of sequencing results for this study) and for each sequencing cycle we determined the fraction of reads that perfectly matched those bases in the known barcode sequence. We identified a decline in sequencing accuracy with each additional sequencing cycle (Figure 2A). As the molecular barcodes are only 20 bases in length and our sequencing primers lie three bases from the beginning of each barcode we are only concerned with the first 23 sequenced bases in this study. On the basis of this assay we determined the false negative rate of sequences that fail to match the barcode for 23 base sequence reads is less than 2%.

To test the sources of variation and the reproducibility of quantitative sequencing of barcodes we investigated the effect of each step in our protocol. Recovery of barcode sequences from genomic DNA requires an initial PCR amplification of the barcodes. As PCR is an exponential process it is conceivable that noise will increase with the number of PCR cycles. Therefore, we investigated the effect of additional PCR cycles on barcode counts by sequencing aliquots removed from a single PCR reaction tube after 15, 20, 25 and 30 cycles of amplification (Figure 2B). We found good correlation between the estimated relative abundance of each barcode by comparing each reaction to a reaction with an additional 5 cycles of PCR. The poorest correlation is between 30 cycles and all previous cycles, which may be a result of PCR reagents becoming limiting after 25 cycles as we found that the total yield (in mass) of DNA from the reaction reached a plateau at 25 cycles (data not shown). We conclude that PCR amplification of barcodes is linear up to 25 cycles under these conditions and the optimal number of cycles, which provides both sufficient yield and linearity of amplification, is 20 PCR cycles.

We found extremely high reproducibility of normalized barcode counts when the same PCR reaction from a complex pool of mutants was sequenced on
two different flow cells (Figure 2C). Finally, we performed replicate DNA preparations, PCR and sequencing reactions of the same heterogeneous samples and confirmed that results are extremely well correlated (Figure 2D). We conclude that our assay is experimentally robust. As we routinely obtained greater than $6 \times 10^6$ individual sequences per sample there are theoretically over 6 orders of magnitude of dynamic range available. We sequenced uptags and downtags from each biological sample in the same lane within a flow cell and for the purposes of analyses we treated uptag and downtag measurements as individual measures of mutant abundance. For each sequencing reaction approximately 25% of reads did not perfectly match a known barcode (TABLE S1). As our sequencing error is less than 2% we assumed that unidentified barcodes were primarily due to errors in the strains’ barcodes themselves as opposed to sequence errors. These data were excluded from further analyses.

**Experimental design for multiplexed survival analysis:** We developed an experimental design that allowed multiplex analysis of individual mutant survival upon defined starvation using quantitative barcode sequencing (Figure 3 and methods). Mixtures of mutant strains were constructed by growing individual mutants on solid rich media and subsequently pooling them. An aliquot of the heterogeneous population was used to inoculate cultures limited for either phosphate or leucine. Population growth ceased 24 hours after inoculation and we define this point as initiation of the starvation phase. At each time point we removed a sample from the population and performed a 24-hour outgrowth in supplemented minimal medium. This step was required to enrich mutants that survive starvation from those that have perished. By performing the identical outgrowth step at every time point, and determining rates of change in abundance in the viable fraction of the population, our design normalizes for any growth rate differences between mutants in minimal complete media.
We used our quantitative sequencing method to determine the change in population composition prior to initiation of starvation. Sequence analysis of the unselected pooled samples identified 7,016 unique barcodes corresponding to 4,497 unique strains. Therefore, sequencing of both barcodes for each strain results in almost complete (93.5%) identification of the expected nonessential haploid gene deletion strains. We compared mutants detected in unselected pooled samples with our initial inocula (t=0) in both nutrient limited cultures. As we performed outgrowth in minimal complete media for each time point in our experiment, including the t=0 point, this analysis identifies those mutants that are able to grow in rich laboratory media but unable to grow in minimal medium. We identified 73 mutant strains that were well measured in the pooled sample but reduced in abundance by more than one hundred fold in the t=0 sample in both the leucine and phosphate starvation experiment (Table 1). Consistent with our expectation, mutants that are unable to survive the outgrowth in minimal media are strongly enriched for the gene ontology (GO) process term “cellular nitrogen compound biosynthetic process” (42 of 74 genes; genome frequency = 4.5%; p=1.7x10^{-35}) and include many of the known auxotrophies that are unable to grow in the absence of nutritional supplementation. In addition, strains mutant for regulation of biosynthetic pathways (GCN3, GCN4), Ras signaling (SRV2), iron-sulfur cluster biogenesis (ISA1, ISA2) and protein and phosphatidylinositol kinase activity important for vacuolar targeting of proteins (VPS15 and VPS34) are purged from the population during this initial selection. Our ability to specifically identify this expected class of mutants in our heterogeneous pool of mutants provided additional validation of the utility of our quantitative barcode sequencing method.

**Survival trends during prolonged nutrient starvation:** We analyzed changes in the diversity of phosphate and leucine starved populations at
each time point during the starvation experiments using quantitative barcode sequencing. First, we counted the number of unique strains detected at each time point (**Figure 4A**). Most strains persist in the population at some level for the first 144 hours following inoculation in both media. The greatest distinction in population diversity was observed at the final two time points. At \( t = 267 \) hours, 78% (3165 of 4033) of strains initially present in the population remained viable in the leucine starvation condition whereas 96% (3948 of 4087) of strains initially present in the phosphate starved population remained viable. This difference was more pronounced after 473 hours at which point only 37% of the initial strains were detected in the leucine starved population (1169 of 4033) and 86% (3528 of 4087) of strains were detected in the phosphate starved population. On average, one strain is lost from the population per hour of phosphate starvation but six strains are lost from the population per hour of leucine starvation. These observations are consistent with starvation of a leucine auxotroph for leucine imposing a much stronger selection on cells than starvation for phosphate.

We performed hierarchical clustering of relative changes in individual mutant abundance during both starvation regimes (**Figure 4B**). For this purpose, the relative abundance of each mutant was determined in the population and expressed as the ratio of abundance at each time point to the abundance of that mutant at \( t = 24 \) hours (plotted as the \( \log_2 \) transformation of this value). Thus, this analysis distinguishes those mutants that increase in representation in the population from those that decrease in representation. In the clustered data we frequently observe the uptag and downtag (separately PCR amplified) co-clustering. Two general trends are apparent in the resulting clustergram. First, most deletion mutants are unchanged in relative abundance throughout phosphate starvation but change in relative abundance to a far greater degree during
leucine starvation. Second, in both starvation regimes the majority of deletion mutants are reduced in relative abundance. This suggests that the typical effect of a deletion mutation on survival is detrimental and a minority of mutants is able to increase in relative abundance through an increased relative survival.

We identified a number of interesting clusters that distinguish mutant survival profiles in the two different conditions and provide qualitative evidence consistent with our expectations. A cluster of mutants appears to be specifically reduced in relative abundance upon starvation for phosphate (Figure 4C). This cluster includes null mutants of PHO4, a transcriptional regulator of the phosphate starvation response, PHO81, the cyclin dependent kinase inhibitor required for inhibition of the PHO80-PHO85 cyclin dependent kinase upon phosphate starvation, and PHO84, which encodes a high affinity inorganic phosphate transporter. We identified a cluster including several mutants that increase in relative abundance when starved for leucine but not phosphate (Figure 4D). This cluster includes the mutants TOR1Δ0 and PPM1Δ0, which we previously recovered as spontaneous loss of function mutants in a genetic screen for increased survival upon leucine starvation (Boer et al. 2008). Loss of TOR1 signaling has been reported to promote longevity in yeast (Powers et al. 2006; Wei et al. 2008). Significantly, our analysis shows that the TOR1Δ0 strain is not altered in relative abundance in the phosphate starved population suggesting that its loss of function does not promote survival in phosphate starvation conditions. A cluster of null mutants is reduced in relative abundance when starved for either phosphate or leucine (Figure 4E) contains several strains mutant for autophagy genes (ATG1, ATG2, ATG3, ATG7, ATG9, ATG12, ATG15, ATG17, ATG18, ATG21). Autophagy, the process by which cells degrade cellular components, is an important process for survival of nutrient starvation (Takeshige et al. 1992). It is noteworthy that we
failed to identify a cluster of mutants that increase in abundance in both phosphate and leucine starvation.

**Quantitation of relative change in individual abundance:** We tested each strain for significant change in relative abundance (compared to the total population) throughout the starvation periods (methods). We determined that 1,333 of 4,337 measured strains (32%) starved for phosphate differed significantly in their relative abundance during the experiment (Table S2). By contrast 3,951 of 4,299 measured mutant strains (92%) starved for leucine were significantly altered in their relative abundance (Table S3). The majority of leucine-starved null mutants decrease in relative abundance (3500 mutants; 88.6%), but 458 (11.6%) mutants increase in relative abundance. For mutants starved for phosphate, 956 (71.7%) decrease in relative abundance and 377 (28.3%) increase in relative abundance.

The majority of null alleles that increase in relative abundance in either leucine or phosphate starvation are non-overlapping. By contrast, an overlapping set of 785 null mutants decreased in relative abundance when starved for either phosphate or leucine. We performed GO term enrichment analysis of mutants that are increased or decreased in relative abundance in either condition or both (Table 2 and methods). We found significant enrichment for several GO terms for genes that are reduced in relative abundance in both starvation conditions that are related to autophagy (both nuclear and mitochondrial) and vacuolar transport. In addition, several GO terms unique to mutants reduced in relative abundance during phosphate starvation are related to mitochondrial function and chromatin. The only GO term we identified associated with increased survival is peroxisomal transport, which is enriched for mutants that increase in relative abundance when starved for leucine.
Quantitation of absolute rates of mutant abundance: Ideally, multiplexed analysis of mutants generates equivalent data to those obtained by testing each mutant individually. For our purposes, experiments performed on individual mutants determine the absolute rate of cell death. We sought to extract this rate from our multiplexed data by estimating the absolute number of each genotype using relative strain abundance and total population viability simultaneously (methods). To test the accuracy of our method of absolute rate estimation we determined the rate of death of a neutral deletion strain, the kanMX-marked deletion of the HO locus, in the mixed population with estimates of the death rate determined for the isogenic strain BY4742 determined in an individual assay. We estimate a death rate for the HO knockout strain in the phosphate starvation regime of 0.22% / hour based on both the uptag and downtag (Figure 6A). The identical death rate is found when BY4742 is starved for phosphate as a pure culture (Figure 6A). When starved for leucine, BY4742 dies at a rate of 2.2%, which is not statistically different from the estimated death rate of the HO knockout strain in the leucine starved population, which we estimate to be 2.88% / hour (uptag value) and 2.77% / hour (downtag value) (Figure 6B). This demonstrates that our combined experimental and analytical methods allow us to obtain absolute rates of death from the pooled experiment that are good estimates of rates obtained by performing starvation experiments for each strain individually.

We fit an over-dispersed Poisson generalized regression model to estimated absolute mutant abundance data for 6,806 unique barcodes corresponding to 4,337 unique strains in the phosphate starvation experiment and 6,730 unique barcodes corresponding to 4,293 unique strains in the leucine starvation experiment. On the basis of this analysis we determined significant death rate estimates (FDR < 5%) for 4,143 different
strains starved for phosphate (Table S4) and 3,591 different strains
starved for leucine and (Table S5). We found good concordance between our
results from the pooled experiments and individual mutant analysis for a
small number of additional tested genotypes in both conditions (Figure
S1).

We used the calculated death rates for each mutant to determine the
distribution of half-lives for mutants starved for phosphate (Figure 6C) or
leucine (Figure 6D) assuming an exponential death rate. The median half-
lives of these distributions are 288.6 hours for mutants starved for
phosphate and 22.8 hours for mutants starved for leucine. The
distributions are widely dispersed consistent with many hundreds of genes
resulting in either increased or decreased survival of differing relative
effect.

Non-randomly distributed mutant classes: We hypothesized that
functionally related mutants important for cell cycle exit, aging and
survival would exhibit similar half-lives when starved for either leucine
or phosphate. Mutants belonging to these gene classes should have half-
lives that are non-randomly distributed within the complete distribution of
half-lives estimated based on our regression model. To test these
predictions we systematically compared the distribution of half-lives for a
priori defined gene sets with the complete distribution of half-lives and
tested the probability that the sub-distribution is drawn randomly from the
overall distribution using the non-parametric Mann-Whitney-Wilcoxon test.

First, we tested gene sets defined by the complete GO annotation for
yeast, for non-random survival behavior upon starvation for phosphate or
leucine. Gene sets were also defined by the GO Slim categorization, which
provides a less granulated classification of genes. We found that genes
annotated to the GO Slim term “mitochondrial organization” are
significantly skewed in their distribution of half-lives when starved for
phosphate (Figure 6A) and leucine (Figure 6B). Mutants annotated to several additional terms related to mitochondrial function are non-randomly distributed in both experiments including mitochondrial inner matrix (GO:0005743), cellular ATP synthesis coupled to proton transport (GO:0015986) and cytochrome-c oxidase activity (GO:0004129) (Table S6). We tested gene sets defined by protein complexes and identified significant results for mitochondrion and mitochondrial ribosome (Table S6). Finally, we tested gene sets defined by high throughput phenotypic analysis (Dudley et al. 2005; Giaever et al. 2002). Consistent with a role for mitochondrial function in survival in prolonged starvation states we find that cells defective for growth on non-fermentable carbon sources have statistically significantly reduced half-lives in both starvation regimes (Table S6). These data strongly implicate mitochondrial function as critical for the survival response to nutrient starvation.

It is possible that oxidative metabolism has a general role in survival as we also find that genes annotated to peroxisome also have reduced half lives when starved for phosphate (Figure 6A). Peroxisomes are organelles that perform fatty acid oxidation and we have previously found that mRNAs encoding components of the peroxisome are increased in expression at slow growth rates (Brauer et al. 2008). Consistent with our analysis of relative death rates, mutants of peroxisomal genes also result in increased half lives when cells are starved for leucine. Although we find multiple lines of evidence for the role of respirative metabolism, we do not find that gene sets defined by metabolic pathways are significantly altered in survival. In fact, lipid-linked oligosaccharide biosynthesis and inositol phosphate biosynthesis are the only non-randomly distributed biosynthetic pathways in phosphate and leucine starvation respectively (Table S6).
In agreement with our analysis of relative death rates, we identified non-random distribution of mutants annotated to the GO term autophagy (GO:0006914) in both phosphate (Figure 6C) and leucine (Figure 6D) starvation. It has been proposed that mRNA translation has a role in replicative aging of yeast cells as cells mutant for ribosomal functions appear to have increased lifespan (STEFFEN et al. 2008). In contrast, we find that genes in the GO term class translation (GO:0006412) are enriched for significantly reduced half lives in both phosphate (Figure 6C) and leucine (Figure 6D) starvations.

We found several cases in which gene sets are non-randomly distributed in phosphate starvation only. In all cases these classes of mutants die faster than wildtype cells. These include mRNA processing (GO:0006397) and mRNA transport (GO:0051028), which appear to have an effect in phosphate starvation (Figure 6E) but not leucine starvation (Figure 6F); gene sets related to chromatin functions including chromatin modification (GO:0016568) (Figure 6G and Figure 6H), the protein complexes histone acetyltransferase (Figure 6G and Figure 6H) and the histone deacetylase and Ino80 complex (Table S6); and the GO Term cytoskeleton (GO:0005856) and GO Slim term microtubule organizing center. The different results for these gene suggests that just as some individual gene deletions have effects in only a single starvation condition different genetic modules may only be important for response to particular nutrient starvations.

We considered that slower growing mutants may have enhanced survival upon encountering starvation as slow growth rate is correlated with increased stress resistance (Lu et al. 2009). Contrary to this possibility we find that mutants that grow slowly in rich media laboratory conditions (GIAEVER et al. 2002) have greatly reduced half lives when starved for phosphate (Figure 6K) and leucine (Figure 6L). Similarly, those mutants that are sensitive to rapamycin, an inhibitor of cell proliferation, have
reduced half lives in phosphate (Figure 6K) and leucine (Figure 6L) starvations. A similar result is observed for the ribosomal inhibitor, cycloheximide (Table S6). Mutants for genes that have been defined as phenotypic capacitors – i.e. deletion of the gene results in increased variance of cell morphological traits - (Levy and Siegal 2008) are non-randomly distributed, and tend to die faster, in phosphate starvation conditions perhaps indicating a requirement for phenotypic uniformity in the complex processes of cell cycle exit, aging and survival.

Previously, we have defined transcripts whose expression level is highly correlated with the growth rate of cells (Brauer et al. 2008). We found little evidence for altered survival of mutants that correspond to growth-rate related transcripts (Table S6). Amongst gene sets defined by global gene expression studies we find that mutants corresponding to those transcripts expressed in the “reductive building” phase of the yeast metabolic cycle (Tu et al. 2005) are the only non-randomly distributed gene set in our data and die faster than wildtype. These mRNAs primarily encode mitochondrial ribosomal components making this observation consistent with our identification of mitochondrial function as critical for response to nutrient starvation.

DISCUSSION

The aim of this study was to determine the contribution of all nonessential genes to the processes of exit from the cell cycle and persistence in a viable state during prolonged starvation. We developed an approach to multiplexed mutant analysis using quantitative sequencing of molecular barcodes to identify the relative abundance of gene deletion mutants in complex mixtures. We performed several control experiments to validate this method and identify the optimal experimental and analytical methods for analyses of these data. Importantly, we have developed a
statistical approach to analyzing barcode counts derived from high throughput sequencing methods that is widely applicable. When combined with accurate determination of population viability our method enabled simultaneous analysis of the absolute rate of survival of ~4,000 null mutants using a single culture. We treated survival as a quantitative trait and tested sets of genes for non-random distribution within the overall distribution enabling a systems-level analysis of genetically defined modules that are active in the complex processes of cell cycle exit, aging and survival during prolonged starvation.

**Cell cycle exit and long-term survival is a complex quantitative trait:** We examined survival upon starvation in two vastly different scenarios. By starving yeast for phosphate we studied the response to a natural limitation. This is presumably a limitation that yeast cells experience in the wild and thus we can expect that genetic networks exist that mediate response to phosphate starvation. This condition is in contrast to our alternate starvation regime: starvation of a leucine auxotroph ($LEU2\Delta 0$) for leucine. This is an entirely laboratory contrived scenario and we have no reason to expect that yeast cells should be able to respond appropriately to leucine starvation. Consistent with this expectation, we observe an order of magnitude difference in the rate of death when populations are starved for leucine compared with phosphate.

Despite the dramatically different selections imposed by these two starvation conditions we identified sets of genes that when mutated impair the response to starvation of both nutrients. We identified autophagy as a cellular process that when impaired results in reduced survival in both starvation conditions. This is consistent with the activation of autophagy by nutrient starvation (TAKESHIGE *et al.* 1992) and its requirement for survival of diverse nutrient starvation conditions. The rapid rate with which autophagy mutants die suggests that this function is required at a
very early stage following cell cycle exit and entry into quiescence. We also determined that strains mutant for mitochondrial function are impaired in their response to starvation for phosphate and leucine. This is, to our knowledge, the first evidence that mitochondrial function is required for survival to phosphate starvation. Thus, whereas proliferative metabolism in yeast may typically involve fermentation, non-proliferative metabolism may generally require respirative metabolism even in the presence of abundant glucose.

We found genetic evidence for several processes that appear to be uniquely important in the case of phosphate starvation. These processes include those related to mRNA processing and transport; chromatin remodeling; as well as microtubule and cytoskeletal functions. These findings are consistent with earlier studies of phosphate starvation. The transcriptional reprogramming of the cell associated with nutrient starvation (Gasch et al. 2000; Saldanha et al. 2004) is likely impaired in cells defective in mRNA related processes consistent with this finding. Regulation of the phosphate starvation response requires chromatin remodeling (Steger et al. 2003) at PHO4 regulated genes, which is consistent with our identification of significantly altered behavior of chromatin remodeling mutants including the INO80 complex. Response to nutrient starvation is likely to involve reorganization of the cytoskeleton (Bauer et al. 1993; Lee et al. 1998) consistent with our identification of reduced survival of this class of mutants upon phosphate starvation.

An interpretation we favor is that mutants with increased survival upon leucine starvation represent cases of genetic suppression and are informative about genes whose function is essential to orderly cell cycle arrest and initiation of a survival program in response to natural starvations. A good example of this is the increased survival upon leucine starvation that is conferred by loss of TOR1 function, which is implicated
in sensing nutrient abundance (Zaman et al., 2008). We suggest that the suppression of starvation lethality of TOR1 and associated genes mimics the required down-regulation of the TOR1 system when nutrition is insufficient. We posit that leucine insufficiency is not detected by the intact TOR system, and thus cells starved for leucine continue to attempt cycling even while starving, and thereby kill themselves (Boer et al., 2009). We identified several additional mutants that confer increased survival upon starvation for leucine. These mutants are potentially informative about gene functions that must be down-regulated upon natural starvations.

Conversely, mutants that reduce survival of leucine-starved cells can be considered cases of genetic enhancement and are informative about genes whose function must be increased during normal cell cycle arrest and survival. These mutants largely overlap with mutants that reduce survival upon phosphate starvation.

Previous studies of chronological aging in yeast may be influenced by starvation for auxotrophic requirements: Yeast is a useful model system for studying aging at a cellular level. The literature in this field is notable for the widespread disagreement between researchers regarding which genes and processes are relevant for either chronological or replicative aging. Our study suggests that many of these discrepancies may be due to a failure to control for the critical relationship between genotype and nutrient limitation. This study reinforces our previous report showing that the first nutrient exhausted in the culture profoundly effects the survival of cells (Boer et al. 2008). Here we have shown that the effect of each null mutation on the death rate differs as a function of which nutrient is depleted first in the media. It has long been known that yeast cells are able to maintain high viability for many hundreds of days when prototrophs are starved for different nutrients (Lillie and Pringle 1980) and the comparatively short lifespan reported for yeast cells under starvation
conditions in different studies (Burtner et al. 2009; Fabrizio et al. 2005; Fabrizio et al. 2001; Longo 2003; Powers et al. 2006; Steffen et al. 2008; Wei et al. 2008) is in clear conflict with these earlier observations. Recently, two papers have described a similar study to this report in which the entire yeast mutant library has been starved in a pooled experiment and assayed using DNA microarrays to determine molecular barcode abundance (Fabrizio et al. 2010; Matecic et al. 2010). In the study by (Matecic et al. 2010) cells were starved in complete media with either 2% or 0.5% glucose. In the former case, the half life of the wildtype strain is around 3 day whereas 0.5% glucose results in a half life of around 10-11 days. In the study by (Fabrizio et al. 2010) cells were starved in synthetic complete media and the heterogeneous population appears to have a half life of around 3-4 days. As with many studies of “chronological aging” these studies made use of strains bearing auxotrophic markers and for which the limiting nutrient (i.e. the nutrient that is first exhausted in the culture) is undefined. Given our previous results (Boer et al. 2008) and the results of this study it is likely that a careful determination of which nutrient limitation is experienced by cells would do much to reconcile conflicting observations in the literature.

It also seems possible that the different responses to natural and unnatural nutrient depletion may underlie some of the discrepancies reported in studies of replicative aging in yeast. In particular, we suggest that the use of low concentrations of glucose as a model for “caloric restriction” (Kaeberlein et al. 2005a; Lin et al. 2002; Murakami et al. 2008) may be confounded by the fact that different concentrations of glucose may alter which nutrient is first exhausted in these media.

The use of auxotrophic strains may effect many studies: Given the profound impact that auxotrophies have on the survival of mutants upon starvation, and the potential unappreciated effect this may have had in
many studies, we believe that extreme caution should be employed for physiological studies that make use of auxotrophic markers in undefined media. Auxotrophies have been found to confound other genetic studies (Chopra et al. 1999; Destrueille et al. 1995) and it seems probable that confusion due to their use may be more pervasive than we expect. Given that the growth rate of chemically complemented strains differs from prototrophic strains (Pronk 2002) and transporters of auxotrophic requirements, such as the uracil permease, are degraded under starvation conditions for nutrients other than uracil (Volland et al. 1994) it seems possible that auxotrophies may generally impact assays that rely on either growth rate or viability in a variety of experimental designs.

**Implications for complex genetic traits:** The classification of heritable phenotypes often entails a somewhat arbitrary division between mutant and non-mutant classes. In reality, all traits exist on a quantitative continuum and our distinction between mutant and non-mutant phenotypes represent an arbitrary, albeit practical, distinction. Quantitative trait mapping in crosses of genetically diverged strains provides a means of identifying the genetic basis of traits (Brem et al. 2002; Rockman and Kruglyak 2006) but these efforts are informative only when genes are polymorphic and the results only detect alleles of strong effect. Ideally, a saturating genetic approach should identify all genes contributing to phenotypic variation and describe their effect size. We have achieved this in our experiment - albeit, for null alleles only - by treating survival to nutrient starvation as a quantitative trait and carefully controlling the environment and genotype. The product of this experimental design is a high-resolution, and near complete, quantitative phenotype-genotype map.

A central question in quantitative genetics is how many genes underlie complex traits. We show that several hundred genes (i.e. a large fraction
of the genome) can contribute to phenotypic variation in response to nutrient starvation. We find it plausible that a similar relationship holds in complex human traits, i.e. that many hundreds of genes are likely to contribute to complex trait variation such as morphological variation or disease risk. This is consistent with the small amount of genetic variance explained in genetic studies of complex traits such as human height (Weedon et al. 2008) and risk for schizophrenia (Consortium et al. 2009). The possible contribution to phenotypic variation of many hundreds of genes may be an underlying source for the hidden heritability problem in human genetic studies (Manolio et al. 2009). The additional complexity contributed by allelic variation, evolution, population history and environmental variation make the genetic dissection of complex traits in any natural population a formidable task.

FIGURE LEGENDS

Figure 1. Survival and physiological parameters for heterogeneous mutant populations during starvation conditions. The entire collection of haploid deletion mutants was starved for either phosphate (+) or leucine (x) in the presence (solid line) or absence (dashed line) of kanamycin for nearly 500 hours following an initial period of 24 hours of batch growth. (A) Survival of replicate populations grown in media of identical composition except for the limiting nutrient. Survival of each population was monitored by determining viability of the population at each time point by counting CFUs on rich media plates. (B) The total number of cells/mL was determined at each time point and remained essentially unchanged throughout the starvation regime. (C) Culture biomass was estimated using a Klett colorimeter and showed a gradual increase for populations starved for phosphate and gradual decrease when populations were starved for leucine. (D) The average cell volume, measured using a Coulter counter, showed a
gradual increase for populations starved for phosphate and a slow decline for populations starved for leucine.

**Figure 2. Development and validation of quantitative barcode sequencing for multiplexed mutant screens.** We tested the error and variance associated with each step of our protocol. **(A)** By sequencing a single barcode we found that 98% of sequences up to sequencing cycle 23 (gray dotted line) perfectly match the expected sequence (n = 2,340,984). **(B)** Additional cycles of PCR introduce minimal variation in the estimated proportions of mutants. The best-correlated estimates of mutant abundance are found between 15-25 PCR cycles (increasingly black values approach a correlation of 1.0; the minimum correlation is 0.94). **(C)** Resequequencing the same PCR product from a complex mixture of mutants on two different flow cells yields highly reproducible results (Pearson correlation = 0.99; n = 3329). **(D)** Complete technical replicates of quantitative barcode sequencing (i.e. independent DNA preparations, PCR and sequencing reactions) are highly reproducible (Pearson correlation = 0.94; n = 3439).

**Figure 3. Experimental design for multiplexed mutant survival analysis using quantitative barcode sequencing.** We constructed normalized pools of the yeast haploid deletion collection by growing individual mutants on rich media (YDP) plates and pooling mutants in liquid YPD for archival purposes. A 1.6mL aliquot of the unselected pooled mutants was used to inoculate (t=0) cultures limited for either phosphate or leucine. The starvation period commenced after 24 hours of culture growth. At each time point we removed a 1mL sample from the culture and expanded the viable sub-population by allowing 24 hours of outgrowth in minimal media. DNA was isolated from the resulting culture and analyzed using quantitative barcode sequencing.

**Figure 4. Population diversity decline and mutant abundance profiles during prolonged starvation.** **(A)** We determined the number of unique
strains identified through barcode sequencing at each time point for populations starved for phosphate (gray bars) or leucine (black bars). (B) Hierarchical clustering of mutant abundance profiles during starvation experiments. We clustered vectors of relative abundance in the population normalized by the abundance of each mutant at t = 24 hours (log₂ transformed). Black indicates that the strain has not changed in abundance. Yellow represents increases in abundance and blue represents decreases in abundance. Failure to detect the strain in the population is indicated by gray. We identified clusters of mutants that were either specifically (C) decreased in relative abundance upon phosphate starvation or (D) increased in relative abundance upon leucine starvation. Several mutants are decreased in relative abundance under both starvation conditions including (E) a cluster including several autophagy gene mutants.

**Figure 5.** Quantitative analysis of absolute death rates during prolonged starvation. We calculated the absolute rate of death for mutants using measurements of population viability and estimates of relative strain abundance using quantitative barcode sequencing of uptags (circles) and downtags (triangles) for the putatively neutral HOΔ0 allele starved for phosphate (A) and leucine (B). The data presented are barcode counts normalized between all uptag or downtag sequencing results. A value of 1 was added to all normalized barcode counts prior to log₂ transformation. The rate of death was determined using a generalized linear model for uptag (long dash line) and downtag (short dash line) data. These rates were compared to rates calculated from independent data obtained for the isogenic strain BY4742 (crosses) subjected starvation in pure cultures. Data are presented as viable cells / μL. We calculated death rates for all mutants in each starvation conditions and converted these values to half-lives for all barcode data that yielded a significant death rate (FDR
The distribution of half-lives for mutants starved for phosphate is centered around 289 hours (C) and 22.8 hours (D) for leucine starved mutants. The half life of the HOΔ0 strain when starved for phosphate (blue dotted line) or leucine (red dotted line in both histograms) is shown for reference.

**FIGURE 6.** Functional gene modules altering survival to nutrient starvation. The distributions of half lives for subsets of genes defined by different methods of categorization were compared with the overall distribution of half-lives (white bars) in each experiment shown. (A) Phosphate starvation, mitochondrion organization (blue, Go Slim term, n = 281, p = 5.88 x 10^-41), peroxisome organization (red, GO:0007031, n = 27, p = 0.00013) (B) Leucine starvation, mitochondrion organization (blue, Go Slim term, n = 276, p = 9.23 x 10^-14), peroxisome organization (red, GO:0007031, n = 25, p = 4.63 x10^-5) (C) Phosphate starvation, autophagy (blue, GO:0006914, n = 51, p = 2.0 x 10^-13), translation (red, GO:0006412, n = 305, p = 1.46 x 10^-12) (D) Leucine starvation, autophagy (blue, GO:0006914, n = 50, p = 0.00014), translation (red, GO:0006412, n = 301, p = 2.33 x 10^-11) (E) Phosphate starvation, mRNA processing (blue, GO:0006397, n = 91, p = 3.38 x 10^-11), mRNA transport (red, GO:0051028, n = 42, p = 6.45 x 10^-5) (F) Leucine starvation, mRNA processing (blue, GO:0006397, n = 91, p = 0.87), mRNA transport (red, GO:0051028, n = 40, p = 0.87) (G) Phosphate starvation, chromatin modification (blue, GO:0016568, n = 89, p = 2.86 x 10^-5), histone acetyltransferase complex (red, SGD defined protein complex, n = 41, p = 8.82 x 10^-5) (H) Leucine starvation, chromatin modification (blue, GO:0016568, n = 90, p = 0.032), histone acetyltransferase complex (red, SGD defined protein complex, n = 40, p = 0.16) (I) Phosphate starvation, cytoskeleton (blue, GO:0005856, n = 151, p = 2.03 x 10^-5), microtubule organizing center (red, SGD defined protein complex, n = 30, p = 3.16 x 10^-5) (J) Leucine starvation, cytoskeleton (blue, GO:0005856, n =
148, p = 0.14), microtubule organizing center (red, SGD defined protein complex, n = 30, p = 0.75) (K) Phosphate starvation, slow growth in YPD (blue, defined by (Giaever et al. 2002), n = 637, p = 2.46 x 10^{-13}), impaired growth in rapamycin (red, defined by (Dudley et al. 2005), n = 137, p = 1.16 x 10^{-19}) (L) Leucine starvation, slow growth in YPD (blue, defined by (Giaever et al. 2002), n = 619, p = 3.04 x 10^{-16}), impaired growth in rapamycin (red, defined by (Dudley et al. 2005), n = 132, p = 0.00058) (M) Phosphate starvation, MRPL10 cluster of the yeast metabolic cycle (blue, defined in (Tu et al. 2005), n = 54, p = 1.62 x 10^{-16}) (N) Leucine starvation, MRPL10 cluster of the yeast metabolic cycle (blue, defined in (Tu et al. 2005), n = 53, p = 4.8 x 10^{-6}).
Table 1. **Mutants purged from the population due to outgrowth in minimal media.** We compared the mutants detected in the mixed populations constructed by growth on rich media with mutants in the leucine and phosphate limited cultures immediately following inoculation (t=0 hours) and outgrowth for 24 hours in complete minimal media. This class of mutants primarily includes auxotrophic mutants that were not chemically complemented during the outgrowth.

<table>
<thead>
<tr>
<th>MUTANTS</th>
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<tbody>
<tr>
<td>AAT2, ACO1, ADE1, ADE12, ADE2, ADE3, ADE4, ADE5, ADE6, ADE8, ARG1, ARG2, ARG3, ARG4, ARG5,6, ARO2, ATE1, BRO1, BUD25, BUD32, CAF17, CDC40, CPA1, CPA2, CYS4, DOC1, ECM29, GCN3, GCN4, GND1, GON7, GRX5, HFI1, HOM3, HOM6, ILV1, ISA1, ISA2, MET6, MET7, MOT2, ORT1, PEP7, PHA2, POS5, RIB4, RNR1, RPL27A, SER1, SER2, SHM2, SLA2, SPT7, SRV2, STB5, THR1, THR4, TRP1, TRP2, TRP3, TRP4, TRP5, TYR1, VPS15, VPS34, YDR008C, YER068C-A, YER091C-A, YIL039W, YKR041W, YOR302W, YOR364W, ZWF1</td>
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*YER068C-A is a dubious ORF encoded on the strand opposite ARG5,6. YOR302W is a uORF that regulates translation of CPA1.*

Table 2. **GO term enrichment analysis of mutants that are significantly altered in relative abundance during starvation period.** We tested genes for GO term enrichment that were either significantly increased or decreased in abundance in either experiment as determined using a regression model and an FDR of 5%. We also identified those genes that were significantly altered in relative abundance in both experiments. P-values are Bonferroni adjusted to account for multiple testing.
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<th>Starvation Condition</th>
<th>Relative abundance</th>
<th>GO term enrichment</th>
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<td>Phosphate</td>
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<tr>
<td></td>
<td>Decreased</td>
<td><strong>Process ontology:</strong></td>
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<tr>
<td></td>
<td></td>
<td>- vacuolar transport  $1.87 \times 10^{-11}$</td>
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<td>- piecemeal microautophagy of nucleus  $1.60 \times 10^{-11}$</td>
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**Leucine**

**Increased**

**Function ontology:**
- transcription factor activity 0.005178439

**Process ontology:**
- peroxisomal transport 0.003125654

**Decreased**

**Function ontology:**
- transmembrane transporter activity $2.23 \times 10^{-07}$
- phosphoric ester hydrolase activity 0.006618899

**Process ontology:**
- catabolic process $1.57 \times 10^{-07}$
- response to stimulus $7.18 \times 10^{-07}$
- response to chemical stimulus $1.40 \times 10^{-06}$
- cellular catabolic process $6.39 \times 10^{-06}$
- organic acid transport $2.75 \times 10^{-05}$
- carboxylic acid transport $2.83 \times 10^{-05}$
- vacuolar protein catabolic process $7.17 \times 10^{-05}$
- reproductive cellular process 0.000117342
- transport 0.0000118658
- ion transport 0.000149536
- establishment of localization 0.0000152932
- filamentous growth 0.000206233
- localization 0.000206468
- cell differentiation 0.000211337
- reproductive process in single-celled organism 0.000466565
- amine transport 0.001303683
- cellular response to chemical stimulus 0.002486978
- biological regulation 0.002673032
- sporulation resulting in formation of a cellular spore 0.004605798
- sporulation 0.004605798
- sexual reproduction 0.005551172
- autophagy 0.0077015
- transmembrane transport 0.008679718

**Component ontology:**
- intrinsic to membrane $2.36 \times 10^{-18}$
- integral to membrane $2.18 \times 10^{-16}$
- membrane part $5.20 \times 10^{-15}$
- membrane $4.05 \times 10^{-14}$
- vacuole 0.000112893
- fungal-type cell wall 0.00657447

**Overlap**

**Increased**

**Function ontology:**
- carnitine O-acetyltransferase activity 0.004722998

**Process ontology:**
- strand invasion 0.005073972

**Decreased**

**Process ontology:**
- vacuolar transport $3.08 \times 10^{-11}$
- piecemeal microautophagy of nucleus $1.22 \times 10^{-09}$
- autophagy $3.69 \times 10^{-08}$
- cellular catabolic process $3.70 \times 10^{-08}$
- protein targeting to vacuole $1.50 \times 10^{-07}$
- microautophagy $1.58 \times 10^{-07}$
- catabolic process $1.87 \times 10^{-07}$
- CVT pathway $3.92 \times 10^{-06}$
- mitochondrion degradation $3.27 \times 10^{-05}$
- macroautophagy $7.48 \times 10^{-05}$
- chromatin modification 0.000224553
- macromolecule catabolic process 0.000389849
- chromatin organization 0.000451277
- organelle organization 0.000617029
- cellular macromolecule catabolic process 0.001004526
- response to starvation 0.001575439
cellular response to nutrient levels 0.001891053
response to nutrient levels 0.00224056
cellular response to starvation 0.002809263
cellular response to extracellular stimulus 0.004995302
cellular response to external stimulus 0.004995302
post-translational protein modification 0.005010949
response to external stimulus 0.005630996
response to extracellular stimulus 0.005630996
late endosome to vacuole transport 0.008312095

Component ontology:

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SUPPLEMENTARY FILES

Figure S1. Comparison of absolute death rate estimates inferred from pooled experiment for uptags (circles) and downtags (triangles) with individual mutant analysis in pure cultures (crosses). Data are presented for ATG10Δ0 starved for (A) phosphate and (B) leucine and PEX13Δ0 starved for (C) phosphate and (D) leucine. Barcode counts are the normalized as described in (methods). Pure culture counts are number of viable cells/μL.

Table S1. Summary of sequencing experiments. All sequencing data that passed filters in the Illumina software pipeline were analyzed and processed with custom Perl scripts.
Table S2. Modeled relative survival rates for all mutants starved for phosphate. We performed regression analysis for 6806 barcodes corresponding to 4337 unique genes. We calculated significant death rates for 1333 genes at an FDR of 5%.

Table S3. Modeled relative survival rates for all mutants starved for leucine. We performed regression analysis for 6740 barcodes corresponding to 4299 unique genes. We calculated significant death rates for 3951 genes at an FDR of 5%.

Table S4. Modeled absolute survival rates for all mutants starved for phosphate. We performed regression analysis for 6806 barcodes corresponding to 4337 unique genes. We calculated significant death rates for 4143 genes at an FDR of 5%.

Table S5. Modeled absolute survival rates for all mutants starved for leucine. We performed regression analysis for 6730 barcodes corresponding to 4239 unique genes. We calculated significant death rates for 3591 genes at an FDR of 5%.

Table S6. Complete results for gene class enrichment analysis. We tested gene classes defined by GO terms, GO slim terms, protein complexes, biochemical pathways, high throughput phenotypic studies and gene expression studies for significant non-random distributions of half lives.

ACKNOWLEDGEMENTS

We thank Jessica Buckles and Donna Storton for technical assistance with Illumina Sequencing. Research was supported by the NIGMS Center for Quantitative Biology (GM 071508), NIH grants to DB (GM 046406) and JS (HG 002913) and start up funds from NYU to DG.
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high-throughput quantitative analysis of yeast chronological life


individual mutants growth on solid rich media

mixed population in YPD

phosphate limited

leucine limited

24 hour outgrowth in minimal media

t = 0 hr

t = 473 hr

quantitative barcode sequencing

24 hour outgrowth in minimal media

t = 0 hr

t = 473 hr

quantitative barcode sequencing
unique strains detected

Phosphate starvation  Leucine starvation

hours post-inoculation

A

B

C

D

E