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Genome-wide analysis of longevity in nutrient-deprived *Saccharomyces cerevisiae* reveals importance of recycling in maintaining cell viability

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Summary

Although typically cosseted in the laboratory with constant temperatures and plentiful nutrients, microbes are frequently exposed to much more stressful conditions in their natural environments where survival and competitive fitness depend upon both growth rate when conditions are favourable and on persistence in a viable and recoverable state when they are not. In order to determine the role of genetic heterogeneity in environmental fitness we present a novel approach that combines the power of fluorescence-activated cell sorting with barcode microarray analysis and apply this to determining the importance of every gene in the *Saccharomyces cerevisiae* genome in a high-throughput, genome-wide fitness screen. We have grown > 6000 heterozygous mutants together and exposed them to a starvation stress before using fluorescence-activated cell sorting to identify and isolate those individual cells that have not survived the stress applied. Barcode array analysis of the sorted and total populations reveals the importance of cellular recycling mechanisms (autophagy, pexophagy and ribosome breakdown) in maintaining cell viability during starvation and provides compelling evidence for an important role for fatty acid degradation in maintaining viability. In addition, we have developed a semi-batch fermentor system that is a more realistic model of environmental fitness than either batch or chemostat culture. Barcode array analysis revealed that arginine biosynthesis was important for fitness in semi-batch culture and modelling of this regime showed that rapid emergence from lag phase led to greatly increased fitness. One hundred and twenty-five strains with deletions in unclassified proteins were identified as being over-represented in the sorted fraction, while 27 unclassified proteins caused a haploinsufficient phenotype in semi-batch culture. These methods thus provide a screen to identifying other genes and pathways that have a role in maintaining cell viability.

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Introduction

When compared with the laboratory environment, conditions found in nature can place considerable stress on the microbial cell with resistance and susceptibility depending on an interaction of genetic and physiological factors. Elucidation of genetic determination of stress survival is impossible in mixed, natural populations and difficult in the majority of environmental organisms. *Saccharomyces cerevisiae* is an important model microorganism and subsequent to its systematic sequencing in 1996 (Goffeau *et al.*, 1996) an international consortium undertook the systematic deletion of (almost) all of the ~6000 open reading frames (ORFs) identified (Baudin *et al.*, 1993; Wach *et al.*, 1994; Winzeler *et al.*, 1999) making this organism an ideal tool for investigations of impact of gene deletion on fitness. Deletion of each gene was accompanied by the introduction of two genetic barcodes that allow the ~6000 yeast strains to be discriminated from one another. This approach permits the performance of experiments in which representative cells of all of the deletion strains can be competed together in a single culture allowing identification of the 'winners' and 'losers'

by the presence, absence and/or relative abundance of the different deletant strains as determined by the level of hybridization of their respective barcodes to specific features on an Affymetrix, Genflex Tag 3 chip.

In this study we have used heterozygous strains as this allows competition between all ~6000 strains, including those with deletions of essential genes, which would not have been possible with the haploid and diploid homozygous deletant collections (Matecic *et al*., 2010). This approach also permits investigation of more subtle gene dosage effects on fitness and three distinct phenotypes can result – haplosufficient (where deletion of one copy of the gene has no observed effect under the conditions tested), haploinsufficient (where there is a decrease in fitness when one copy is deleted) and haploproficient (where deletion of one copy results in an increase in fitness). Using this approach we have previously described the identification and importance of genes that exert a high degree of control over growth rate in *S. cerevisiae* during competitions in chemostats (Delneri *et al*., 2008). Chemostat competition has a long history in microbiology (Novick and Szilard, 1950) and has the advantage of providing a controlled and reproducible environment and, in particular, allows comparisons between different growth media at the same growth rate. However, outside of the laboratory yeast will seldom experience such a controlled, constant environment. The chemostat approach allows selection of strains, and thereby identification of genes, which affects fitness defined as growth rate under constant nutrient supply and indeed growth rate, is also used as a proxy for fitness in genetic studies (MacLean and Buckling, 2009). However, in the environment microbes must also be able to survive fluctuating nutrient levels and periods of multiple nutrient deprivation (starvation stress).

Gene deletions that lead to increased fitness under a given environmental condition can thus be identified straightforwardly by selection in chemostats. Here we address the more difficult task of identifying the strains that were haploinsufficient for maintenance of viability in *S. cerevisiae*. To this end we have developed a novel method based upon flow cytometry and fluorescence-activated cell sorting (FACS) (Davey and Kell, 1996) to isolate strains with reduced viability from a nutrient-deprived population. Although, in the strictest sense, proliferation is the only true confirmation of viability in microbial systems (Kell *et al*., 1998), many dye-based rapid methods have been developed that give a good predictive indication of the subsequent ability of a cell to proliferate (Davey *et al*., 2004). Yeast cells that had been exposed to a starvation stress were dual-stained for viability and analysed by flow cytometry. The population of cells with the most reduced viability was sorted (physically separated). In order to determine the abundance of each deletant strain within the sorted and total populations, DNA was extracted from the samples and used to identify strains that showed significantly increased proportional representation in the sorted (low viability) population when compared with the total population.

While nutrient deprivation is an important environmental stress, yeast may be expected to experience a range of other stresses and resistance to a combination of these will be important in determining overall fitness. Differences in gene expression between wild type and domesticated strains of *S. cerevisiae* have been highlighted by Kuthan and colleagues (2003) and thus the use of a more challenging environment (compared with standard, controlled laboratory media and conditions) may be expected to apply more diverse and realistic selective pressures to a yeast population. In nature, *S. cerevisiae* is commonly found as a minor component of the grape microbiota, being more frequently found on berries with damaged surfaces (Mortimer and Polsinelli, 1999) although it has been postulated that the relationship between plant and yeast may be beneficial to both, with ethanol consumption modifying frugivore behaviour and thus increasing seed dispersal (Dudley, 2004). Yeast cells are vectored between grapes by insects (Shetty, 2006) and experience fluctuations in nutrient availability, increasing ethanol concentration and growth-related acidification of their environment. To model this, we have developed a semi-batch fermentor system in which the cells undergo a standard batch growth curve for 48 h and are then held in stationary phase for 48 h before dilution of the culture with fresh medium. This process is repeated for the duration of the experiment to allow selection of strains capable of both surviving and competing well in these conditions. Thus, for a particular strain to exhibit a high level of fitness under this regime, it must grow quickly, persist in a recoverable state in stationary phase and emerge quickly from lag phase once nutrients become available. We have competed barcoded heterozygous deletion mutants in semi-batch cultures using grape juice as the medium [as a proxy for the high-sugar, low-nutrient environment where *S. cerevisiae* is typically found in nature (Mortimer and Johnston, 1986)]. Those deletant strains, which were lost, or greatly reduced in proportion, may be described as having a haploinsufficient phenotype. In order to quantify and visualize the selection pressure exerted by the semi-batch fermentor regime we present a fermentation model (Sinclair *et al*., 1987) consisting of two competing populations of cells.

Thus our aim was to identify genes that were haploinsufficient for survival of environmentally relevant conditions of nutrient limitation. Through this work we have identified genes whose deletion affected fitness negatively under nutrient deprivation. The distribution of the strains between the different gene functional classes

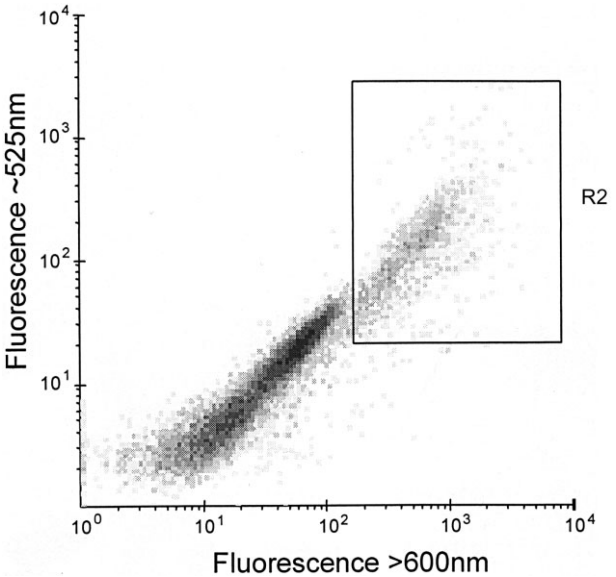

Fig. 1. Flow cytometric analysis of *S. cerevisiae* stained with $DiBAC_4(3)$ and propidium iodide. The region R2 indicates dead cells and was sorted from the total population before analysis on the barcode microarray.

highlights a number of cellular mechanisms that are significantly over-represented among those strains with reduced fitness. We show that in a fluctuating environment lag phase is a more important measure of fitness than growth rate.

Results

Flow cytometry, sorting and barcode analysis

The pooled heterozygous diploid collection of gene deletion mutants (Winzeler *et al*., 1999) of *S. cerevisiae* was subjected to a low nutrient stress as described in *Experimental procedures*. Following growth to stationary phase (48 h) on grape juice yeast were incubated for a further 7 days before sampling and staining with propidium iodide (PI) and $DiBAC_4(3)$. Flow cytometric analysis revealed a major population (~ 90%) with low levels of fluorescence and a minor population (~ 10%) with elevated fluorescence in both the red (> 630 nm) and green (525 nm) channels (Fig. 1). The region labelled R2 in Fig. 1 was used as a sort gate, i.e. cells with properties measured in this range were separated into a collection tube while other cells were passed through the flow cytometer into the waste reservoir.

The frequency of each of the yeast deletion mutants in the sorted (dead) fraction of yeast from R2 (Fig. 1) was compared with the unsorted (total) population. The relative abundance of each mutant was determined, over-representation of a particular mutant's DNA in the sorted (R2) population compared with the total population was predicted to be indicative of its reduced fitness (ability to survive the low-nutrient stress) relative to the other mutants. A list of ORFs was identified with normalized intensities in the sorted fraction of at least 1.1 × that of the total population (representing an increase of at least 10% in population density). The data were filtered further by selecting only those ORFs where there was correlation of results between the (machine) replicate spots on each chip and also between biological replicates analysed on separate chips. These criteria resulted in a list of 735 ORFs (see Table S1) where deletion of a single copy led to a reduced fitness under the conditions applied (haploinsufficiency). Essential genes accounted for 120 out of the 735 ORFs and these would not have been identified if homozygous or haploid strains had been used.

Functional analysis of genes resulting in short-lived phenotypes

Analysis of the 735 highlighted ORFs using the Munich Information Centre for Protein Sequences (MIPS) 'Functional Distribution of Gene Lists' (Ruepp *et al*., 2004) search tool at http://mips.gsf.de/proj/funcatDB/search_main_frame.html (accessed April 2011) (Guldener *et al*., 2005; Mewes *et al*., 2011) revealed that a number of functional categories were significantly over-represented (at $P < 0.05$) in the sorted fraction (see Table 1). As the number of ORFs within the *S. cerevisiae* genome that are assigned to each process varies between the categories, it is the difference between the expected proportion of strains with that process (i.e. the proportion of that process in the total population of strains) compared with the observed proportion with that process in the sample that is significant, rather than the actual number of ORFs. In addition, the ratios of signal intensities between the sorted and total populations were calculated for each barcode and those strains that were most consistently highly over-represented in the sorted fraction are shown in Table 2.

Verification of short-lived phenotypes

The top 15 deletants listed in Table 2 and the wild-type BY4743 background strain were analysed using methylene blue staining (Jones, 1987; Davey and Hexley, 2011) following starvation (see *Experimental procedures*). Under these conditions samples of the wild type were 99–100% viable, while, with the exception of YDR482c (98% viable), the samples from the deletant strains contained between 10% and 80% blue (dead) cells. The most reduced viabilities were seen with YMR298W and YDR373W (both ~ 80% dead) and YGR201C (~ 77% dead).

Table 1. MIPS functional categories that were over-represented in the sorted (reduced viability) population.

Functional category	Number of ORFs (sample)	Percentage of ORFs (sample) (%)	Number of ORFs (genome)	Percentage of ORFs (genome) (%)	*P*-value
20.01 transported compounds (substrates)	97	13.1	585	9.54	3.63×10^{-4}
12 protein synthesis	82	11.1	480	7.83	4.13×10^{-4}
20.03.02.03 antiporter	6	0.81	14	0.22	3.76×10^{-3}
20.03.02.03.01 proton driven antiporter	4	0.54	7	0.11	5.35×10^{-3}
42.19 peroxisome	10	1.35	35	0.57	6.48×10^{-3}
20.01.21 RNA transport	18	2.44	86	1.4	0.012
42 biogenesis of cellular components	124	16.8	862	14	0.013
01.01.09.05.01 biosynthesis of tyrosine	2	0.27	2	0.03	0.014
20.03.02 carrier (electrochemical potential-driven transport)	6	0.81	18	0.29	0.015
12.01 ribosome biogenesis	50	6.79	310	5.05	0.016
42.01 cell wall	36	4.89	214	3.49	0.021
20.01.10 protein transport	25	3.39	140	2.28	0.026
20.09.07.06 post Golgi transport	3	0.4	6	0.09	0.026
20.01.15 electron transport	16	2.17	83	1.35	0.036
01.01.09.04.01 biosynthesis of phenylalanine	2	0.27	3	0.04	0.040
10.03.04 nuclear and chromosomal cycle	20	2.71	111	1.81	0.040
12.01.01 ribosomal proteins	39	5.29	246	4.01	0.040
40.20 cell aging	7	0.95	28	0.45	0.043
16 protein with binding function or cofactor requirement (structural or catalytic)	143	19.4	1049	17.1	0.044
01.01.09.04 metabolism of phenylalanine	5	0.67	17	0.27	0.044
10.03.04.05 chromosome segregation/division	12	1.63	59	0.96	0.045
16.03 nucleic acid binding	51	6.92	341	5.56	0.054

Table 2. Deletant strains that were enriched more than twofold by sorting.

ORF	Gene	Average increase in population density in the sorted fraction	Description [from MIPS (http://mips.gsf.de/genre/proj/yeast/) or Stanford (http://www.yeastgenome.org/) databases]
YGR049W	*SCM4*	43.1	Cdc4 suppressor
YGR044C	*RME1*	16.9	Zinc-finger transcription factor
YGR201C	–	13.0	Strong similarity to translation elongation factor eEF1 alpha chain Cam1p
YLR197W	*SIK1*	10.4	Protein involved in pre-rRNA processing
YDR373W	*FRQ1*	9.2	Regulator of phosphatidylinositol-4-OH kinase protein
YGR038W	*ORM1*	8.3	Unfolded protein response protein
YGR055W	*MUP1*	7.6	High-affinity methionine permease
YGR043C	–	7.4	Putative transaldolase
YNL268W	*LYP1*	7.2	Lysine permease
YDL163W	–	7.0	Dubious ORF
YDL157C	–	6.9	Protein of unknown function
YFL030W	*AGX1*	6.5	Alanine – glyoxylate aminotransferase
YBR289W	*SNF5*	6.5	Component of SWI/SNF transcription activator complex
YMR298W	*LIP1*	6.1	Ceramide synthase subunit
YDR482C	*CWC21*	5.4	Component of complex containing Cef1p, putatively involved in pre-mRNA splicing
YPR037C	*ERV2*	5.3	Flavin-linked sulfhydryl oxidase
YER074W	*RPS24A*	5.3	40 s small subunit ribosomal protein S24.e
YDR404C	*RPB7*	5.2	DNA-directed RNA polymerase II, 19 KD subunit
YGR193C	*PDX1*	4.8	Pyruvate dehydrogenase complex protein X
YDR334W	*SWR1*	4.8	DEAH-box protein, putative RNA helicase
YER061C	*CEM1*	4.2	Mitochondrial β-keto-acyl-ACP synthase
YDL009C	–	3.9	Dubious ORF
YMR117C	*SPC24*	3.4	Outer kinetochore protein – part of Ndc80p complex
YDR242W	*AMD2*	3.3	Amidase
YKL113C	*RAD27*	3.1	ssDNA endonuclease and 5′-3′ exonuclease
YDR380W	*ARO10*	3.1	Phenylpyruvate decarboxylase
YMR102C	–	3.0	Protein of unknown function

The table only shows those strains that were enriched > twofold in both experiments and where there was agreement between all of the spots representing the ORF on the barcode array. Shaded rows indicate essential ORFs.

Table 3. MIPS functional categories that were over-represented among those deletant strains identified as having a haploinsufficient phenotype during semi-batch fermentation.

Functional category	Number of ORFs (sample)	Percentage of ORFs (sample) (%)	Number of ORFs (genome)	Percentage of ORFs (genome) (%)	*P*-value
11.04.03.05 3′ end processing	3	2.38	22	0.35	9.82×10^{-3}
01.20.17.01 metabolism of nonprotein amino acids	2	1.58	10	0.16	0.017
01.20.33 metabolism of secondary products derived from L-tryptophan	1	0.79	1	0.01	0.021
01.01.03.05.01 biosynthesis of arginine	2	1.58	12	0.19	0.024
16.21 complex cofactor/cosubstrate/vitamine binding	4	3.17	58	0.94	0.031
16.21.07 NAD/NADP binding	3	2.38	36	0.58	0.037
01.03.10 metabolism of cyclic and unusual nucleotides	2	1.58	17	0.27	0.047

Determination of fitness in a fluctuating environment

We extended the environmental fitness model using a semi-batch fermentor system (see *Experimental procedures*) in which all ~ 6000 heterozygous diploid mutants were grown for 48 h, held in stationary phase for 48 h and then diluted with fresh medium to permit further growth. This process was used to select strains capable of growing quickly, persisting in a recoverable state in stationary phase and emerging quickly from lag phase. Haplosufficiency in this system is manifested by an increase in the proportion of a particular strain over time. Conversely, strains with reduced fitness will decrease as a proportion of the total population and in extreme cases may be lost from the fermentor altogether. Barcode analysis at three time points [end of first batch cycle, middle and end of the experiment over 39 generations (862 h)] was conducted with all samples being taken just before dilution with fresh medium. This revealed 134 strains (for full list see Table S2) that had reduced in proportion (haploinsufficient phenotype). Using this model, strains that remain at the same proportion of the total population will have a subpopulation increase (growth rate) of 0. Haploinsufficent strains have a negative 'relative' growth rate (i.e. growth rate lower than the mean for the whole population). Strains were only classified as haploinsufficient if the negative growth rate was less than one standard deviation of the mean and corresponded to $q < 0.05$. The *q*-value represents an estimate of the false discovery rate, and is calculated from the *P*-value (statistical significance) while taking into account the issue of multiple testing (Quackenbush, 2001). As before, the 'Functional Distribution of Gene Lists' search tool revealed a number of functional categories were over-represented in the list of genes with reduced fitness (Table 3).

Modelling fitness in a fluctuating environment

In order to determine the effect of parameters such as division time and lag phase duration on the competitive fitness of *S. cerevisiae* in the semi-batch environment, we devised a model as described in *Experimental procedures*. This allows two populations to be competed against each other. Varying μmax, lag phase, inoculation population size and K_s for nitrogen uptake within literature-reported ranges for *S. cerevisiae* showed length of lag phase to be a key determinant in fitness within the semi-batch fermentor system. Figure 2 shows results of a simulated competition between two strains that are identical except that the lag phase of one is 15 min and the lag phase of the other is 100 min. As can be seen, the strain with the longer lag phase is rapidly outcompeted and would be expected to fall to < 10% of the population in the course of the semi-batch regime reported above.

Comparison with previous genome-wide starvation studies in haploid S. cerevisiae

While individual studies can yield valuable data on survival of low-nutrient stress applied in a particular way and in a given genetic background, identification of deletant strains that have reduced viability under a range of conditions is of wider interest. Consequently, the data reported here were compared with those from two other very recent studies (Fabrizio *et al*., 2010; Matecic *et al*., 2010). However, these used haploid yeast, and therefore only considered non-essential genes (rather than the true genome-wide analyses reported here) and used calorie restriction rather than low levels of nitrogen to induce starvation conditions. Additionally, the methodology for identification of reduced fitness depended upon their failure to grow on plates (Matecic *et al*., 2010) or in broth (Fabrizio *et al*., 2010) to enrich for viable yeast. These approaches will also select against damaged cells that may nevertheless recover, and against strains that grow slowly. Nevertheless, comparison of the results from these experiments with those conducted here revealed 91 deletant strains that showed a reduced viability phenotype in two or more studies (see Fig. 3).

Discussion

Propidium iodide has been widely used for viability determination on the basis that it is excluded by the intact

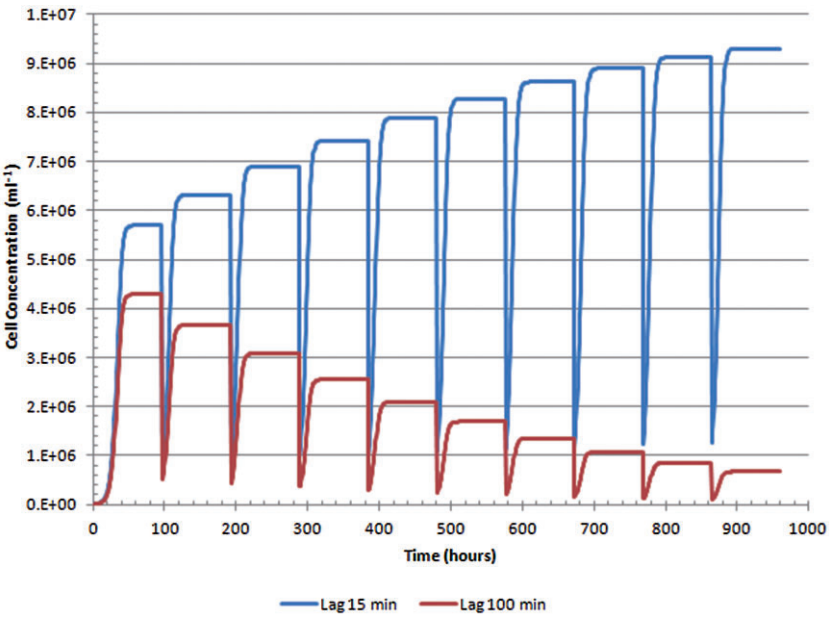

Fig. 2. A simulation of competition between yeast strains with different lag phase duration. Even though the lag phases are short compared with the time spent in exponential growth there is a powerful influence on fitness.

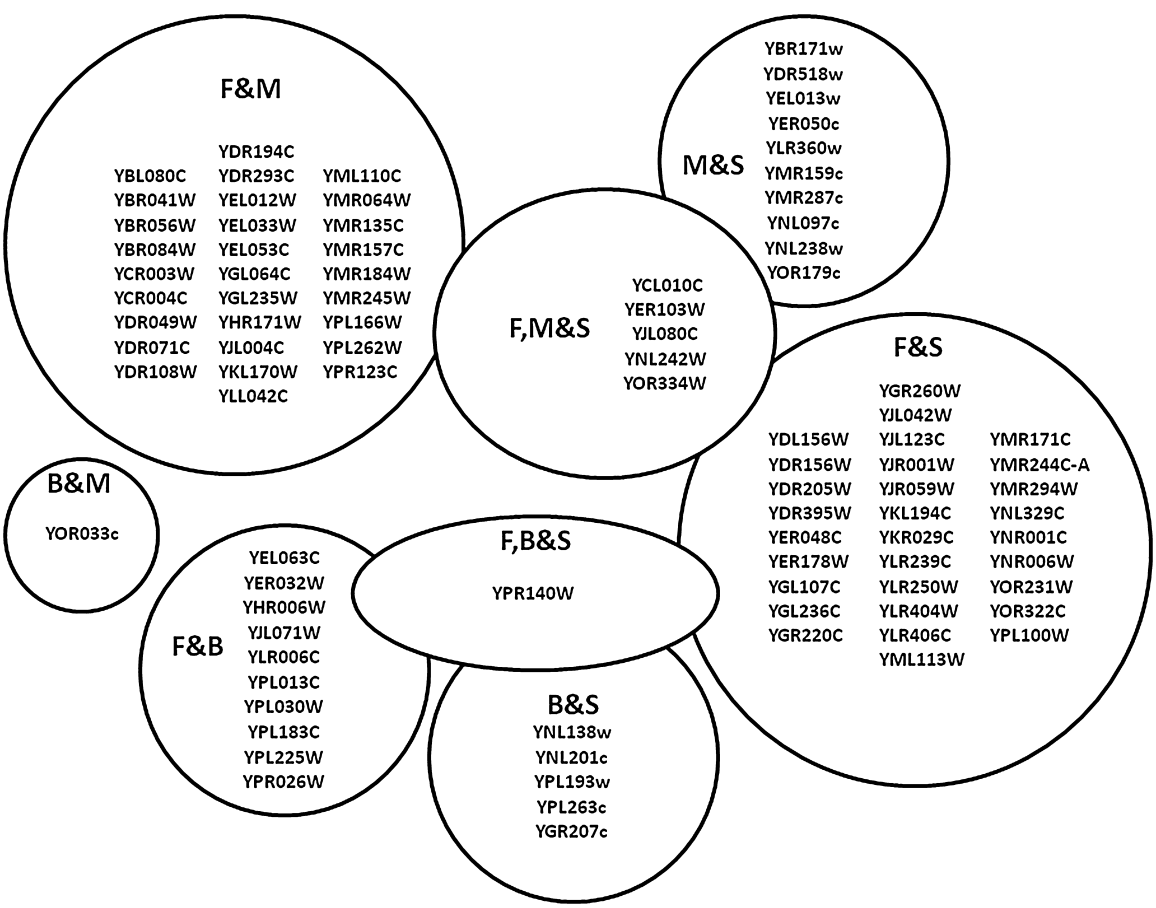

Fig. 3. Comparison of data obtained in this study to genes previously identified as important for survival during nutrient limitation. F, Fabrizio (Fabrizio *et al*., 2010); M, Matecic (Matecic *et al*., 2010); S, sorted fraction; B, semi-batch selected strains (both this study).

membrane of viable animal cells (Jones and Senft, 1985), bacteria (Assunção *et al*., 2006) and fungi including *S. cerevisiae* (Deere *et al*., 1998; Achilles *et al*., 2006). On entering the cell, PI binds to nucleic acids and its fluorescence is enhanced and thus cells with damaged membranes (dead) fluoresce red while those with intact membranes (alive) do not. It is generally considered that once the membrane of a cell is sufficiently damaged to allow the propidium ion to enter, the cell has passed the point of no return and can be described as non-viable (Davey and Kell, 1996); however, recently we have shown that removal of stress may allow a small proportion of cells to recover their permeability barrier (Davey and Hexley, 2011). With this in mind we additionally stained cells with $DiBAC_4(3)$, which enters cells with de-energized membranes (Davey *et al*., 2004) causing them to be green fluorescent. It has previously been shown [e.g. (Van Zandycke *et al*., 2003)] that on staining with PI and $DiBAC_4(3)$ cells that fluoresced both red and green were dead. Using this approach approximately 10% of the cells were phenotypically distinct from the main population and barcode analysis was used to establish that genetic heterogeneity contributed to this.

In the absence of extracellular nutrients, recycling of cell contents and components via selective degradation pathways offers a route for obtaining the nutrients that are essential for maintenance of the viable state. It may thus be hypothesized that heterozygosity of genes required for these recycling pathways should reduce survival under these conditions. Pairwise comparison of population density in the total and sorted (reduced viability) populations revealed that a number of functional categories were over-represented in the sorted fraction (Table 1); as a Bonferroni correction for multiple hypothesis testing (Broadhurst and Kell, 2006) has not been applied we restrict our discussion to the categories where $P \leq 0.03$.

As shown in Table 1, deletion of a single copy of genes in the category ribosome biogenesis (many of which are genes that encode ribosomal proteins) in the heterozygous background resulted in a reduced ability to survive the starvation stress. Having a reduced copy number of genes for certain ribosomal proteins appears to lead to a higher rate of cell death during nutrient deprivation at the end of batch growth. It has previously been shown (Warner, 1999) that the transcription of ribosomal protein genes is regulated by cell growth rate and that amino acid starvation results in a decrease in transcription of these genes. It has been suggested that yeast are capable of sensing impending nutrient limitation and responding to this by degrading existing ribosomes (Ju and Warner, 1994). Thus, cells with a reduced ribosome number have less energy and resources stored in this form to mitigate nutrient limitation. Furthermore, the most over-represented (haploinsufficient) category in the sorted population was 'transported compounds (substrates)' and, in particular, two of the subcategories of this – 'RNA transport' and 'protein transport'. Deletion of *puf4* (a member of the protein transport subcategory) has been shown to affect the stability of transcripts encoding ribosomal proteins and ribosome biogenesis factors (Grigull *et al*., 2004) and the Puf4 protein has a role in regulation of ribosomes in response to starvation (Foat *et al*., 2005). Thus, evidence from both ribosomal protein encoding genes and genes for proteins that regulate ribosome biogenesis support an important role for ribosomal recycling in survival during starvation stress in *S. cerevisiae*.

Ten genes associated with the peroxisome were identified (see *Supporting information*) as being present in a higher proportion in the sorted (low viability) population. This included *PEX3*, which is required for stability of the peroxisome (Hettema *et al*., 2000) and other genes whose products form the peroxisome. Peroxisomes perform a variety of metabolic functions in eukaryotic cells; they contain enzymes that serve in oxidative utilization of specific carbon and nitrogen sources and, in particular, are the sites of fatty acid degradation. Dysfunction of a peroxisomal enzyme or loss of the peroxisome can cause severe defects and lead to a number of serious diseases in humans (e.g. Zellweger syndrome). During stationary phase, cells continue to metabolize, albeit at a reduced rate compared with exponentially growing cells. Although yeast storage carbohydrates (glycogen and trehalose) decrease in concentration during starvation, surprisingly long-term viability in stationary phase does not always correlate with successful accumulation of high levels of these compounds (Sillje *et al*., 1999). In view of this it has been hypothesized (Gray *et al*., 2004) that β-oxidation of fatty acids in the peroxisome may supply energy for survival under starvation conditions. Our detection of a large number of deletants lacking a copy of peroxisome genes indicates that fatty acid degradation may indeed provide an important source of metabolic energy in the nutrient-deprived cell.

We found that deletion of YNL242W (*ATG2*) caused reduced viability on starvation, Atg2p has a role in both autophagy and pexophagy (degradation of the peroxisome) with null mutants being blocked in both (http://db.yeastgenome.org accessed April 2011). Autophagy is a process in which portions of cytoplasm are sequestered and delivered to the lysosome-like vacuole where they are degraded, and recycled under stress conditions such as starvation (Klionsky *et al*., 2003). Specifically, in the context of growth in grape juice (a nitrogen-limited medium), autophagy has been implicated in survival of nitrogen stress (Kohda *et al*., 2007). YBL078c (*ATG8*) is essential for autophagy and our results indicate that deletion of one copy led to increased cell death on starvation. Cebollero and Gonzalez (2006) found that deletion of this

gene in a haploid strain caused levels of Ald6p to remain constant during nutrient starvation, while in the wild-type Ald6p levels declined leading to initiation of autophagy. The role of orthologues of yeast ATG genes in protein turnover is now being recognized in higher eukaryotes (Thompson *et al*., 2005).

Mutations of YNL238w (*KEX2*) (Oluwatosin and Kane, 1998), YKL080w (*VMA5*) (Stevens and Forgac, 1997), YEL051w (*VMA8*) (Stevens and Forgac, 1997), YOR270c (*VPH1*) (Manolson *et al*., 1992) and YKR007w (*MEH1*) (Gao *et al*., 2005) have been shown to cause loss of vacuolar acidification, which would reduce the efficiency of autophagy (Thumm, 2000) and these genes were highlighted in our experiments. Deletion of YKR042w (*UTH1*) has been shown to interfere with biogenesis of mitochondria and *uth1*Δ cells have lower levels of cytochromes aa_3, *c*, and *b*, and the enzyme citrate synthase. It has been reported that mutation in *UTH1* leads to a longer lifespan (Bitterman *et al*., 2003) and better viability during very short-term (15 h) starvation (Kissova *et al*., 2004). However, the data presented here show that in the hemizygous state, longer starvation periods result in significantly reduced fitness of this deletant. Kissova and colleagues (2004) showed that Uth1p is involved in the autophagic degradation of mitochondria (mitophagy) and that the absence of this protein prevented the degradation process. Reduced ability to remove selected mitochondria (Lemasters, 2005) and to recycle their component parts may contribute to cell aging and cell death.

Analysis of changes in population density of individual strains in the semi-batch competitions also identified functional categories that were over-represented in the list of haploinsufficient ORFs (Table 3). The category '3′ end processing' (a subcategory of 'mRNA processing' and ultimately of the 'transcription' category) was most significantly ($P < 0.01$) over-represented. ORFs belonging to this category included the essential genes YAL043c (*PTA1*) (O' Connor and Peebles, 1992) and YKL059c (*MPE1*), which, together form part of the cleavage/polyadenylation factor (CPF) complex (Nedea *et al*., 2003). The third gene identified as haploinsufficient within this category, YER032w (*FIR1*), interacts with the product of the *REF2* gene, which is another component of the CPF complex. The function of CPF is in the formation of messenger RNA 3′ ends, an important step in maturation of eukaryotic mRNAs and essential for gene expression.

Two of the haploinsufficient ORFs, YER069w (*ARG5,6*) and YJL071w (*ARG2*), appear in both the 'metabolism of nonprotein amino acids' and the 'biosynthesis of arginine' subcategories of metabolism. These genes form a metabolon (Abadjieva *et al*., 2001), i.e. they associate in a protein complex that is essential to arginine synthase activity and allows their coordinated feedback regulation controlling the first two steps in arginine biosynthesis (Pauwels *et al*., 2003). Arginine is the most abundant yeast-assimilable amino acid in grape juice (Austin and Butzke, 2000) and is also stored in the yeast vacuole. Presence of arginine would be expected to repress transcription of genes involved in arginine synthesis. However, as grape juice is a nitrogen-limited (~ 200 mg l^{-1}) medium, arginine will be an important growth substrate and its concentration will fall during the growth phase of each semi-batch cycle. Strains with impaired arginine synthesis will thus be haploinsufficient under N-limitation and were identified as having reduced fitness during semi-batch competition in grape juice.

Hemizygous deletion of four ORFs in the 'complex cofactor/cosubstrate/vitamin binding' category led to a haploinsufficient phenotype in the semi-batch competition. These are YHR176w (*FMO1*) and three ORFs that are in the NAD/NADP binding subcategory of this grouping. *FMO1* is not essential to cell viability because other enzymes can generate a significant fraction of the oxidizing equivalents required by the cell. However, it is vital to the yeast response to reductive stress (Suh *et al*., 1999) where it uses molecular O_2 and NADPH to oxidize glutathione and other thiol compounds that are found in grape juice (Park *et al*., 2000). *FMO1* provides a large fraction of the oxidizing equivalents necessary for proper folding of disulphide bond-containing proteins. The likely reduction in concentration of the enzyme in the hemizygous state may reduce correct folding of proteins, under the conditions provided, leading to the haploinsufficient phenotype. Modelling of the semi-batch regime revealed that fitness was characterized by a short lag phase, genetic changes that lead to cellular damage during nutrient limitation would be expected to increase recovery time. Protocols have been developed for the automated screening of phenotypic growth characteristics (Warringer and Blomberg, 2003) and in the future such data may form useful additions to yeast databases [http://mips.helmholtz-muenchen.de/genre/proj/yeast/ (Guldener *et al*., 2005; Mewes *et al*., 2011) and http://www.yeastgenome.org/ (Engel *et al*., 2010) and research community wikis http://wiki.yeastgenome.org/index.php/Main_Page].

A comparison of the lists of gene deletions obtained from the barcode arrays of the low-viability sorted fraction to previous studies (Fabrizio *et al*., 2010; Matecic *et al*., 2010), revealed five genes that were highlighted as having a significant role in maintaining viability in the non-proliferating state in all three studies (Fig. 3). Of particular note are YER103W (*SSA4*), which is a chaperone belonging to the highly conserved HSP70 heat shock protein family (Werner-Washburne *et al*., 1987), YJL080C (*SCP160*), which has previously been shown to have a role in maintenance of viability (Wintersberger *et al*.,

1995) and YNL242W (*ATG2*), which has been discussed above in relation to its role in autophagy.

In conclusion, we have shown that the use of FACS to isolate subpopulations for subsequent analysis on barcode arrays is appropriate as a genome-wide screen for the identification of ORFs that have a role in the response of yeast to starvation stress. In particular, the data presented provide evidence of the important role played by recycling mechanisms in maintaining *S. cerevisiae* in a viable state during nutrient starvation. We also present a semi-batch fermentation regime, which provides a system of applying selective pressures that may be found in the natural environment of *S. cerevisiae* that are not normally encountered in the laboratory. Together these methods provide a novel approach for assessing and understanding environmental fitness. Interestingly, 125 unclassified proteins were identified as being over-represented in the sorted (reduced viability) fraction, while 27 unclassified proteins were identified as causing a haploinsufficient phenotype in semi-batch culture (see *Supporting information*). Further investigations will be required to confirm whether these proteins have a role in maintaining cell viability during starvation. A fuller understanding of the role of individual genes in determining fitness in this important model organism will have implications far beyond the scope of this initial study and may also help to elucidate the function of some of those proteins that do not currently have an assigned biological role.

Experimental procedures

Organism

The complete collection of heterozygous diploid deletion strains of *S. cerevisiae* BY4743 (*MAT**a**/α his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 lys2Δ0/LYS2 MET15/met15Δ0 ura3Δ0/ura3Δ0*) was used. This resource has been widely used and is available commercially from Open Biosystems, ATCC and others – see http://www-sequence.stanford.edu/group/yeast_deletion_project/faqs.html. These were pooled as described previously (Delneri *et al*., 2008) and contained a total of ~ 5.5×10^7 cells ml^{-1} (determined by flow cytometry).

Growth conditions

A 100 μl volume (~ 5.5×10^6 yeast cells) of the pool of heterozygous deletant strains was inoculated into 500 ml of white grape juice (100% pure pressed white grape juice; Sunpride, Somerset, UK) in a fermentor. The contents of the fermentor were stirred continuously at 300 r.p.m. on a magnetic stirrer. Temperature was maintained at 24°C ± 2°C. pH was not controlled as growth-related acidification is a natural environmental stress that yeast approaching stationary phase would be expected to experience. The pH of the grape juice before inoculation was 3.1 and did not fall below 2.85. Stationary phase (judged via plate counts and total counts performed by flow cytometry) was reached after 48 h. After a further 48 h 440 ml (88%) of the yeast suspension was harvested into a capped 500 ml bottle; this sample was incubated at 22°C without agitation for a further 7 days before FACS analysis (see below). The remaining yeast suspension was diluted by the addition of 440 ml of fresh grape juice and this procedure was repeated nine times at 96 h intervals to allow selection to take place. In order to verify the results the whole experimental procedure was repeated such that two true biological replicates were analysed.

Samples from the semi-batch fermentor that were to be used directly for barcode analysis consisted of 2 ml aliquots of yeast suspension. These were removed from the fermentor just before the harvesting and dilution procedure. These aliquots were pelleted in a bench top centrifuge at 14 000 r.p.m. for 10 min, resuspended in 1 ml sterile water and frozen at –80°C until required.

Flow cytometry and FACS

Following starvation of the yeast as described above, a 1 ml sample of yeast was centrifuged at 14 000 r.p.m. for 5 min. The supernatant was discarded and the pellet resuspended in 2 ml 50 mM phosphate buffer (pH 7) containing 6.5 μg ml^{-1} PI (Sigma) and 4.75 μg ml^{-1} bis-(1,3-dibarbituric acid)-trimethine oxonol ($DiBAC_4(3)$) (Molecular Probes, now part of Invitrogen). The sample was incubated in the dark for 20 min.

All flow cytometric analyses were performed using a Coulter Epics Elite flow cytometer as described previously (Davey *et al*., 1999). A rectangular sort region defining the cells with highest fluorescence from both stains was defined and cells were collected into a sterile tube. For barcode array analysis approximately 250 000 cells were collected. The sorted sample was frozen at –80°C.

Preparation of samples for barcode array analysis

Yeast suspensions were defrosted on ice and cells harvested by centrifugation for 10 min at 7500 r.p.m. and resuspended in 600 μl sorbitol buffer [1 M sorbitol, 100 mM EDTA, 14 mM β-mercaptoethanol (all Fisher)] and 20 U of lyticase (Sigma-Aldrich) was added to each sample to lyse the cell walls during a 1 h incubation at 30°C. After lysis the spheroplasts were pelleted by centrifugation for 10 min at 3000 r.p.m. DNA was then extracted using the Qiagen DNeasy Tissue Kit (Qiagen, West Sussex, UK). The primers used for the amplification of the barcodes and the hybridization protocol have been described previously (Winzeler *et al*., 1999). Barcode analysis provided data in the form of an intensity for each spot on the microarray. The relative signal intensity of the spots on the barcode array is proportional to the quantity of each strain present in the original sample. The intensities recorded on each microarray chip were normalized to the total barcode intensity to allow for variation between arrays, and spots with intensity values below background were excluded from further analysis. Each ORF is represented by at least two spots on the chip (the vast majority are represented by more than 2).

Methylene blue staining

For rapid analysis of viability using light microscopy methylene blue staining was used. A stock solution was prepared according to the protocol of Jones (Jones, 1987) at a concentration of 225.6 μg ml^{-1}. Cells were stained in a ratio 1:2:20 of yeast suspension: methylene blue solution: phosphate buffer at room temperature (22°C). Between 200 and 400 cells were manually counted by light microscopy for each sample. Unstained cells and slightly grey-blue coloured cells were scored as alive, whereas cells that appeared deep blue were scored as dead.

Development of competition model

The mathematical model was implemented in the visual declarative modelling language Simile (Simulistics, Midlothian, UK). This consisted of two competing populations of cells utilizing the same limiting nitrogen source under a fluctuating semi-batch regime that mirrored the experiments described above. Each time the fermentor contents were diluted the cells entered a lag phase before exponential growth. The numerical integration used an Euler integrator with a step size on 0.001 h being used for all the simulations.

The equations used in the model were:

$$r_{s1} = \mu_{max1}\ ([N]/(k_{s1} + [N]))$$

$$r_{s2} = \mu_{max2}\ ([N]/(k_{s2} + [N]))$$

$$dB_1/dt = r_{s1}\, B_1$$

$$dB_2/dt = r_{s2}\, B_2$$

$$d[N]/dt = -r_{s1}\, B_1\, Y_1 - r_{s2}\, B_2\, Y_2$$

Subscripts 1 and 2 refer to the two competing cell populations, r_s is the specific growth rate in kg new cells/kg cells/h (i.e. /h), μ_{max} is the maximum specific growth rate in kg new cells/kg cells/h (i.e. /h) and [N] is the concentration of yeast available nitrogen in kg N/m^3 of fermentation medium. k_s is the [N] when r_s equals half μ_{max} (in kg N/m^3 of fermentation medium), B is the cellular biomass concentration in kg cells/m^3 of fermentation medium and Y is the yield factor in kg N used/kg biomass produced.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Table S1. List of all strains showing reduced fitness during starvation.

Table S2. List of the strains showing haploinsufficiency during semi-batch growth.

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