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Identification and characterization of high-flux-control genes of yeast through competition analyses in continuous cultures

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Using competition experiments in continuous cultures grown in different nutrient environments (glucose limited, ammonium limited, phosphate limited and white grape juice), we identified genes that show haploinsufficiency phenotypes (reduced growth rate when hemizygous) or haploproficiency phenotypes (increased growth rate when hemizygous). Haploproficient genes (815, 1,194, 733 and 654 in glucose-limited, ammonium-limited, phosphate-limited and white grape juice environments, respectively) frequently show that phenotype in a specific environmental context. For instance, genes encoding components of the ubiquitination pathway or the proteasome show haploproficiency in nitrogen-limited conditions where protein conservation may be beneficial. Haploinsufficiency is more likely to be observed in all environments, as is the case with genes determining polar growth of the cell. Haploproficient genes seem randomly distributed in the genome, whereas haploinsufficient genes (685, 765, 1,277 and 217 in glucose-limited, ammonium-limited, phosphate-limited and white grape juice environments, respectively) are over-represented on chromosome III. This chromosome determines a yeast's mating type, and the concentration of haploinsufficient genes there may be a mechanism to prevent its loss.

A major aim of systems biology is the construction of a mathematical model of the eukaryotic cell that has both predictive and explanatory power. For both technical and conceptual reasons, the first such models are likely to be of the unicellular eukaryote *Saccharomyces cerevisiae* (baker's yeast). A top-down approach to the construction of such a model would be to identify a subset of cellular components that exert a major degree of control over cell growth and use them to populate a coarse-grained model of the entire cell[1]. To achieve this, we are adopting the concepts of metabolic control analysis (MCA)[2,3]. This approach requires two complementary initial experiments to be carried out. In one, the impact of changes in flux (growth rate) on the direct and indirect products of gene action is measured[4]; in the other, the concentrations of the gene products are altered, and the impact on growth rate is measured.

Here we report experiments in this second category, in which a population of yeast cells comprising heterozygous (hemizygous) deletion mutants[5] for each of the organism's protein-encoding genes[6] were competed with one another in continuous cultures that were grown either in a chemically defined minimal medium or in white grape juice (representing a 'natural' environment[7]). For the defined media, the growth rate–determining nutrient was glucose,

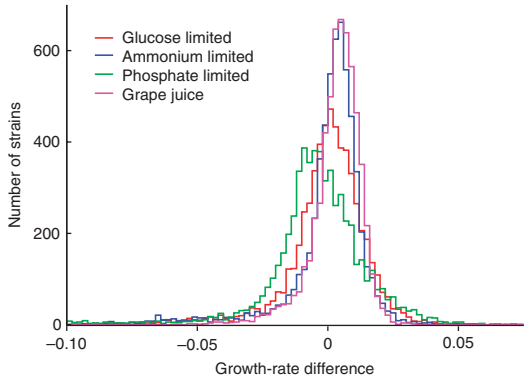

Figure 1 Distribution of growth rate differences in glucose-limited medium (red), ammonium-limited medium (blue), phosphate-limited medium (green) and grape juice (pink). These growth rate differences are relative to the imposed dilution rate of 0.1 h^{-1}.

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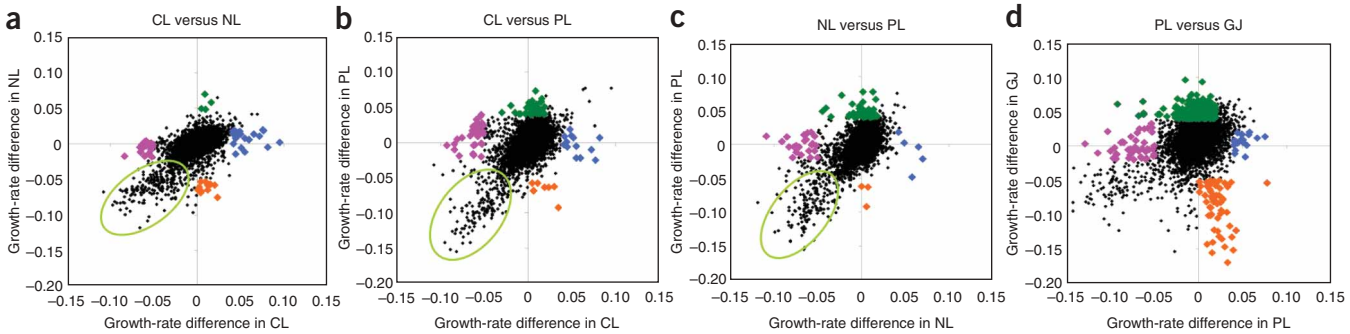


Figure 2 Comparison of haploinsufficient and proficient phenotypes in the different media. (**a**–**d**) Pairwise comparisons among glucose-limited, ammonium-limited and phosphate-limited cultures (**a**,**b** and **c**) and between grape juice and phosphate-limited cultures (**d**). The colored area represents genes that show context-dependent haploinsufficiency (magenta and orange) or haploproficiency (dark green or blue). The light green oval shows the tight correlation between haploinsufficient genes in the three chemically defined media; this is not apparent in the comparison between phosphate-limited and grape juice cultures (similar data were obtained comparing glucose-limited with grape juice cultures and ammonium-limited with grape juice cultures; data not shown).

ammonium or phosphate. In hemizygous diploids, the concentration of the protein encoded by the gene in the haploid state should be about half that found in wild-type diploids. For those genes that encode proteins exerting a high degree of control over growth rate (in the parlance of MCA, proteins with high flux-control coefficients), the concentration of the protein may be insufficient to sustain growth at the wild-type rate. Classically, such genes are said to have a haploinsufficiency phenotype[8].

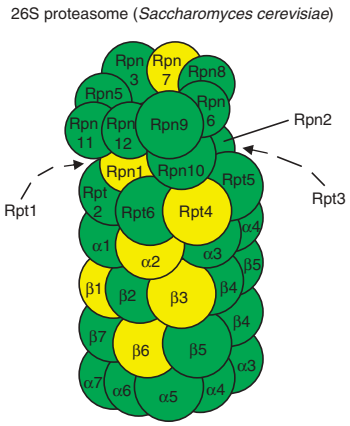
Continuous cultures have two advantages over batch cultures in showing the quantitative phenotypes associated with haploinsufficiency. First, continuous cultures provide greater sensitivity, because the competition may continue for a larger number of generations and hence reveal smaller differences in growth rate. Second, the data from continuous cultures should be more reproducible, as chemostats provide a constant growth rate and environment at steady state. In practice, the period of competition should not exceed 50 generations; otherwise, spontaneous mutations may complicate the analysis[9]. In our experiments, the population of heterozygous mutants was grown in batch for 24 h and then switched into continuous culture, where it took approximately 42 h to reach a steady state, which was maintained for 30 generations of continuous growth. Samples were taken just before the switch to continuous culture, at the point when the cells reached the steady state, and after 18–22 and 26–30 generations in the steady state. We ran parallel chemostats for each nutrient limitation and duplicate hybridizations on each sample. We identified strains that showed a significant change in their proportions in the growing population when compared to that in the starting population. The significance threshold was chosen to give a false-discovery rate of 1% (see Methods and **Supplementary Methods** online), as this would produce a sufficiently large pool of candidate genes to allow functional categories to be identified using Gene Ontology[10].

We did not detect 240 of the deletants in the pooled inoculum (**Supplementary Table 1** online); for 94 of these mutants, this was probably a result of known errors in their TAG sequences[11]. After 24 h growth, a further 137 strains disappeared in at least one nutrient condition; 21 of these carried erroneous TAGs[11]. The remaining 116 strains lost during batch culture (**Supplementary Table 2** online) may identify genes with severe haploinsufficiency phenotypes. Twenty-six strains were lost from the glucose-limited, ammonium-limited and phosphate-limited cultures but were maintained in grape juice (**Supplementary Table 3** online). Ten deletants showed severe haploinsufficiency under all four conditions; their deleted genes included no essential genes and three ORFs of undetermined function (YER139C, YGR130C and YLL029W). Three were mutants that had been shown previously to have reduced fitness in rich medium (YPD) in homozygosis (*aft2*, *pho81*, YER139C) or heterozygosis (*pho81*)[12,13].

Continuous cultures are capable of revealing more subtle haploinsufficiency phenotypes than batch cultures, and minimal media present more stringent selection conditions than rich media. Thus it was unsurprising that our investigation uncovered more haploinsufficient genes than previous studies in which the population of mutants was grown in batch cultures in rich (YPD) medium. We found that approximately 12% of genes showed a haploinsufficiency phenotype in glucose-limited and ammonium-limited cultures, whereas 20% showed such a phenotype in phosphate-limited cultures (complete list in **Supplementary Table 4** online). This contrasts with the 3% of genes showing a haploinsufficiency phenotype when mutants were grown in YPD batch cultures[13]. Our data for grape juice cultures revealed just 3.8% of hemizygotes to be haploinsufficient (**Supplementary Table 4**), a value much closer to that obtained with YPD. Over all, essential genes (as previously defined[12]) were not significantly over-represented in our set of haploinsufficient genes, although there was some significant over-representation ($P < 0.01$) of essential genes in the set showing haploinsufficiency in ammonium-limited cultures.

We grew our continuous cultures at a dilution rate of 0.1 h^{-1}, which is equivalent to a generation time of 6.9 h or about one-third the maximum growth rate for the defined media. In all four growth conditions, we found that a significant proportion of hemizygous mutants increased their relative numbers in the population. Such mutants represented approximately 10%–13% of the strains in glucose-limited, phosphate-limited and grape juice cultures, and about 20% of the strains in ammonium-limited cultures (**Fig. 1** and **Supplementary Table 4**). We term this phenomenon haploproficiency, to contrast it with haploinsufficiency. This phenotype has not been recognized previously, presumably because in earlier studies, yeast cells were already growing at nearly their maximum rate in a rich medium[12,13] (**Supplementary Note** online).

The rate at which individual hemizygous deletants decrease (haploinsufficiency) or increase (haploproficiency) their proportions in the population is shown in **Figure 2a–c** as a series of pairwise comparisons among glucose-limited, ammonium-limited and phosphate-limited cultures. A large number of mutants show a similar haploinsufficiency profile in all three nutrient-limited conditions (tail circled in the bottom left quadrant of the distribution), whereas



Rpn1	Rpn2	Rpn3	Rpn4	Rpn5	Rpn6	
Rpn7	Rpn8	Rpn9	Rpn10	Rpn11	Rpn12	
Rpt1	Rpt2	Rpt3	Rpt4	Rpt5	Rpt6	
α1	α2	α3	α4	α5	α6	α7
β1	β2	β3	β4	β5	β6	β7

Figure 3 The 26S proteasome, adapted from the KEGG pathway database (see URLs section in Methods). Green boxes and subunits represent 24 of 32 proteins encoded by genes that show a haploproficiency phenotype in ammonium-limited cultures. Yellow boxes and subunits represent 8 of 32 proteins encoded by genes that do not show haploproficiency (Rpn4 is a transcription factor for proteasome subunit genes).

haploproficiency is much more likely to be confined to a single nutrient environment. The pairwise comparisons between the population grown in grape juice and those grown in defined media (for example, phosphate-limited medium, as shown in **Fig. 2d**) reveal little commonality in haploinsufficiency profiles, emphasizing the environmental specificity of selection effects.

We used MAPPFinder[14] and GoMiner[15] to associate Gene Ontology terms with all genes that showed haploproficiency in defined media and to detect general or environment-specific trends. Although we found some genes to be haploproficient in all three conditions, Gene Ontology showed little commonality of function among them. However, more genes show a context-dependent haploproficiency, and when we examined their functions, we found some unifying themes. For instance, many genes that show haploproficiency in ammonium-limited cultures share Gene Ontology annotations associated with protein turnover. In particular, hemizygous mutants of 24 of the 32 genes associated with proteasome function have a haploproficient phenotype (**Fig. 3**). Thus, in a nitrogen-poor environment, there is selection for mutants that conserve proteins.

Haploproficient genes might be predicted to include those affecting the uptake of toxic metabolites released by competitors or those that shorten the cell's doubling time. Although we found examples of the latter (for example, *CHK1/chk1* hemizygotes are haploproficient), genes encoding membrane transporters were not over-represented in the haploproficient class (see **Supplementary Note**).

We also attached Gene Ontology terms to genes that showed haploinsufficiency in defined media. Genes associated with the term 'COPI vesicle coat' showed a high degree of haploinsufficiency in all three defined media. This result, combined with the fact that genes involved in Golgi-ER transport were also haploinsufficient, indicates that protein trafficking functions exert a considerable degree of control over growth rate in yeast. Much of the traffic of yeast proteins is directed to bud extension, and three of the four genes encoding components of the protein kinase CKII complex (*CKA2*, *CKB1* and *CKB2*) that controls the polar growth of yeast[16] showed marked haploinsufficiency in all three defined media.

Using Gene Ontology analysis on data from the population grown in grape juice, we found that genes associated with the categories 'nucleobase, nucleoside, nucleotide and nucleic acid transporter', 'pyrimidine transporter' and 'electrochemical potential-driven transporter' showed considerable haploinsufficiency. These included *FUR4*, which encodes a protein involved in uracil transport. Because *fur4 ura3* double mutants show synthetic lethality, we conclude that halving the copy number of *FUR4* in the *ura3/ura3* mutant background of BY4743 compromises fitness in grape juice because it (unlike the defined media) was not supplemented with uracil. BY4743 also carries *leu2/leu2* and *his3/his3* mutations, and so we conclude that grape juice supplies sufficient amino acids, but not pyrimidines. Genes encoding components of the COPI vesicle coat also show haploinsufficiency in grape juice, although at a lower level of significance ($P < 0.02$ versus $P < 0.01$) than was found in defined media.

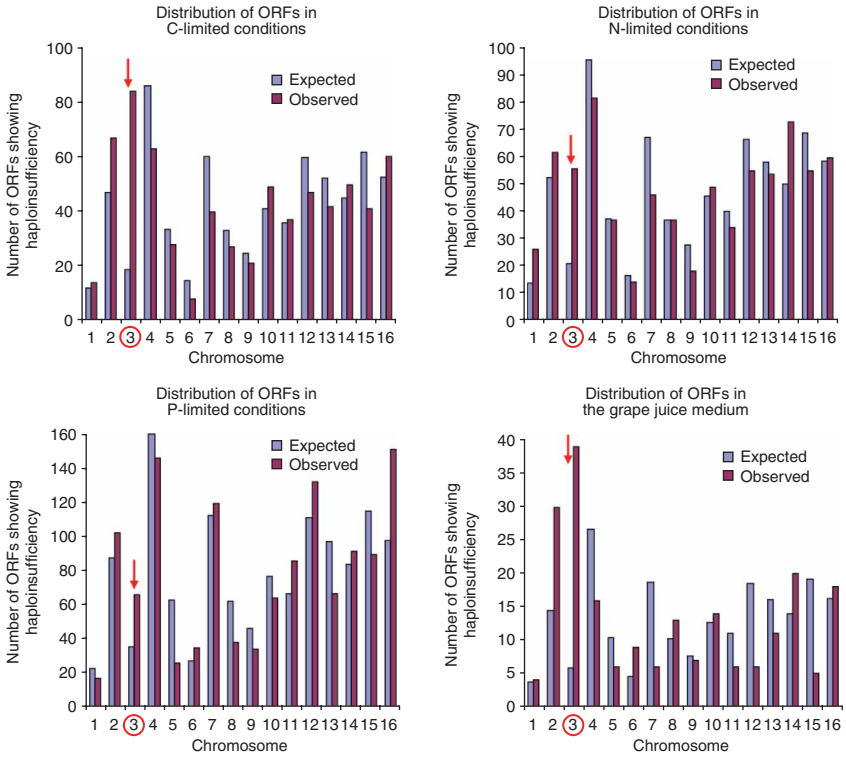


Figure 4 Chromosomal distribution of ORFs showing haploinsufficiency in the different nutritional conditions.

Haploproficient genes identified in grape juice cultures include those in the Gene Ontology categories 'endocytosis' and 'ethanol fermentation'. Reduced endocytosis may protect cells against toxic products from grape juice catabolism, and reduced fermentation favors respiration and reduces ethanol concentrations, thus improving growth yields and viability[17]. Grape juice is poor in nitrogen, and thus we find that genes encoding components of the proteasome complex are haploproficient in this environment, just as they are in ammonium-limited cultures. However, the effect is less pronounced, possibly because the limitation is not as severe in grape juice because it contains more nitrogen, and in a more easily assimilated form, than does ammonium-limited medium (**Supplementary Table 5** online).

We examined the distribution of haploinsufficient and haploproficient genes across the 16 chromosomes of yeast[6]. The distribution of haploproficient genes for any of the four nutrient conditions seemed random. However, genes that have haploinsufficiency phenotypes in each of the conditions tested showed significant over-representation on chromosome III (**Fig. 4** and **Supplementary Figure 1** online). Combining the data from all four media yields a P value of $< 10^{-50}$ for chromosome III, when testing a single unified null hypothesis that haploinsufficient genes are randomly distributed across all chromosomes. This P value is 43 orders of magnitude smaller than that for the next most significant chromosome. It seems unlikely that this result is due to some artifact in strain construction, because the chromosome III deletants were generated in five different laboratories (A. Chu, Stanford University, personal communication).

The *MAT* locus, which is responsible for determining the mating type of *S. cerevisiae*, is found on chromosome III. Haploid cells may be either *MAT**a*** or *MAT*α and are able to mate with cells of the opposite mating type to form a diploid. Diploid *MAT**a**/MAT*α cells are unable to mate but can go through meiosis to produce four haploid spores that are mating competent. We postulate that the accumulation on chromosome III of genes showing haploinsufficiency phenotypes may be the result of intense selection pressure against the loss of one copy of this chromosome from a diploid. Such a loss would have severe consequences for the yeast population, as cells that had lost a copy of chromosome III would be diploid maters. This loss would also disadvantage the individual carrying it because it would prevent the cell from forming spores as a survival device in unfavorable environmental conditions (**Supplementary Methods**).

In this study, we have shown both positive (haploproficient) and negative (haploinsufficient) effects on flux by halving the copy number of yeast protein-encoding genes. The context dependency of these effects on flux has been demonstrated, particularly in the case of haploproficiency. However, we have identified a common core of genes that exert significant control on flux (HFC genes, for high flux control). Previously, in our MCA approach to the systems analysis of yeast[4], we carried out experiments in which we altered growth rate (flux) in chemostat cultures and measured the impact on the transcriptome. That study identified a number of genes whose transcript concentrations were significantly ($Q < 0.01$) upregulated (493) or downregulated (398) with growth rate irrespective of the growth-limiting nutrient. Here, we have shown that 192 and 348 genes show, respectively, haploproficiency and haploinsufficiency in all three defined media. Among these, genes whose transcript levels are under growth-rate control occur no more frequently than expected by chance (26 of 192 and 47 of 348, respectively). Thus, a general rule emerges: genes that are major controllers of growth rate are not themselves subject to growth-rate control. In the jargon of systems biology, this might be termed a 'design rule' for the eukaryotic cell. However, cells are the products of evolution rather than design, and thus the rules of biology may change according to the selection pressures to which an organism is exposed. Given this caveat, we would suggest that this rule about HFC genes holds for nutrient-limited environments. Further studies are required to determine whether it has any greater generality.

METHODS

Strains and oligonucleotides. The heterozygous deletion strains, in the diploid BY4743 background (*MAT**a**/MAT*α *his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 met15Δ0/MET15 LYS2*/lys2Δ0 *ura3Δ0/ura3Δ0*), were obtained from the *Saccharomyces* Deletion Consortium (See URLs section in Methods). For the construction of the heterozygous deletion pool, we grew the strains in YPD with 15% glycerol using 96-well plates at 30 °C until they reached stationary phase (48 h). Using a multichannel pipette, we combined all the hemizygous strains together in a sterile Petri dish. The pool was then divided in 1-ml aliquots, which were stored at –80 °C in YPD with 15% (v/v) glycerol.

Media and growth conditions. YPD medium was prepared as described in Sherman *et al.*[18] We prepared the carbon-, nitrogen- and phosphorus-limiting media as described[19], with the following nutritional supplements: uracil (20 mg/liter), leucine (100 mg/liter) and histidine (20 mg/liter). The grape juice (100% pure pressed white grape juice; Sunpride) was used directly as a growth medium with no further additions.

Competition experiments. Competition experiments in chemostat were carried out as previously described[20] using a small-scale multiple fermenter system (Fedbatch-pro, Das Gip Technology). We inoculated an aliquot (1×10^7 cells) of the hemizygote pool into flasks containing 120 ml of growth medium and allowed them to grow in batch for 24 h at 30 °C, with shaking at 170 r.p.m. The medium feed was then turned on to switch the cultures to continuous growth with a dilution rate of 0.1 h^{-1} and a constant pH of 4.5. Each competition experiment was conducted in two biological replicates for no more than 35 generations; samples were taken at 48-h intervals.

Genomic DNA extraction, Tag amplification and hybridization. We collected a sample of 15 ml from the culture outflow and extracted genomic DNA using the DNA tissue kit (Qiagen). The concentration of DNA in the extract was determined using a Nanodrop (Agilent) device. The primers used for the amplification of the barcodes and the protocol for hybridization to Tag 3 microarrays (Affymetrix) have been previously described[5].

Data analysis. Hybridizations were globally normalized by median centering intensity values from TAGs corresponding to deletion mutants. Log ratios were then calculated between the initial chemostat time point and subsequent time points. This served to eliminate TAG-specific biases and further normalized the data. Growth rates were estimated by robust linear regression on the normalized log ratios. Type I error rates (P values) were estimated by model-based resampling with suitably rescaled residuals. To account for multiple testing, we estimated false-discovery rates (Q values) using the method of Benjamini and Hochberg[21]. We considered growth rates with $Q < 0.01$ as statistically significant and selected the corresponding ORFs for further bioinformatics analysis. A detailed description of the data analysis is provided in **Supplementary Methods**. Intensity values from TAGs not corresponding to deletion mutants were taken as being representative of the background intensity and used to determine the presence or absence of deletion strains in both the pool and batch stages of the experiment (see **Supplementary Methods**).

URLs. *Saccharomyces* Deletion Consortium, http://www-sequence.stanford.edu/group/yeast_deletion_project/deletions3.html; KEGG pathway database, http://www.genome.jp/kegg/.

Note: Supplementary information is available on the Nature Genetics website.

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