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Epistatic buffering of fitness loss in yeast double deletion strains

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Interactions between deleterious mutations have been insufficiently studied[1,2], despite the fact that their strength and direction are critical for understanding the evolution of genetic recombination[3,4] and the buildup of mutational load in populations[5,6]. We compiled a list of 758 yeast gene deletions causing growth defects (from the Munich Information Center for Protein Sequences database and ref. 7). Using BY4741 and BY4742 single-deletion strains, we carried out 639 random crosses and assayed growth curves of the resulting progeny. We show that the maximum growth rate averaged over strains lacking deletions and those with double deletions is higher than that of strains with single deletions, indicating a positive epistatic effect. This tendency is shared by genes belonging to a variety of functional classes. Based on our data and former theoretical work[8–10], we suggest that epistasis is likely to diminish the negative effects of mutations when the ability to produce biomass at high rates contributes significantly to fitness.

Epistasis is positive (in other words, diminishing, antagonistic or buffering) when it enhances the fitness predicted from individual effects of deleterious mutations, and it is negative (that is, reinforcing, synergistic or aggravating) when it decreases fitness[4,5,10]. Positive epistasis alleviates the harm done to an individual by multiple deleterious mutations but tends to lower average fitness of a population at equilibrium. This is so because it reduces variation in fitness and thus reduces the effectiveness of natural selection in removing deleterious mutations. The reverse is true for negative epistasis, which tends to lower average mutational load of a population[5]. Selective elimination of deleterious mutations would be especially effective if negative epistasis were accompanied by genetic recombination[3]. This is why the ubiquity of different forms of genetic recombination, including obligatory sexual reproduction, could conceivably be explained if the rate at which deleterious mutations arose were high and their interplay affected fitness negatively[4].

In earlier experimental measurements of epistasis, neither the number nor the identity of deleterious mutations was usually known. Another problem has been pervasive selection against the least fit individuals, which is likely to bias the distribution of genotypes accessible for fitness assays. The rise and spread of compensatory mutations has also been a concern, especially in long-term experiments. Available data suggest that epistasis may be negative, positive or absent, and it is unclear whether this results from experimental biases or reflects diversity of genetic systems[1]. It has been proposed that positive epistasis is likely to dominate in the simplest systems, like RNA viruses, whereas null or negative epistasis will prevail in more complex ones, but this rule is supported by data on few species, and counterexamples can be found[2]. This leaves the question of the evolutionary role of epistasis largely unanswered, especially because the experiments may not have been sensitive enough to detect small but consistent directional epistasis. The budding yeast is currently the most suitable cellular organism for the study of genetic interactions. A complete collection of strains with single-gene deletions has been created, and the impact of deletions on fitness has been approximated in high-throughput experiments[7,11]. This has created a new situation in which a large set of known mutations can be used in experiments designed specifically to overcome the limitations of earlier studies of epistasis.

We aimed to test all yeast genes whose deletion was known to cause a detectable growth defect (as of 2005). We found 758 gene deletions explicitly annotated as slow growers in the Munich Information Center on Protein Sequence (MIPS) database (see URL in Methods) or qualified as such in well-replicated phenotypic assays of the entire collection of deletions[7]. This set of genes adequately represents the metabolism of the yeast cell. Not only are all major categories of biological processes present, but their frequency distribution approximates that of the entire genome (**Fig. 1**). We chose the nutritionally rich YPD medium and benign temperature of 30 °C for the literature search and experimental environment because most annotations on slow growth refer to these conditions. Notably, the coupling of fluxes in the yeast cell, a feature of metabolism that is likely to affect genetic interactions, is similar in rich and minimal media[12].

Yeast gene deletions are originally marked by an insertion of the *kan* gene, which provides resistance to geneticin. In half of the deletions, we exchanged *kan* for another marker, *nat*, which provides resistance to nourseothricin. Both markers are neutral for fitness[13,14]. We paired differently marked haploid strains at random, mated them to obtain diploids and then sporulated the latter. Yeast asci contain four haploid

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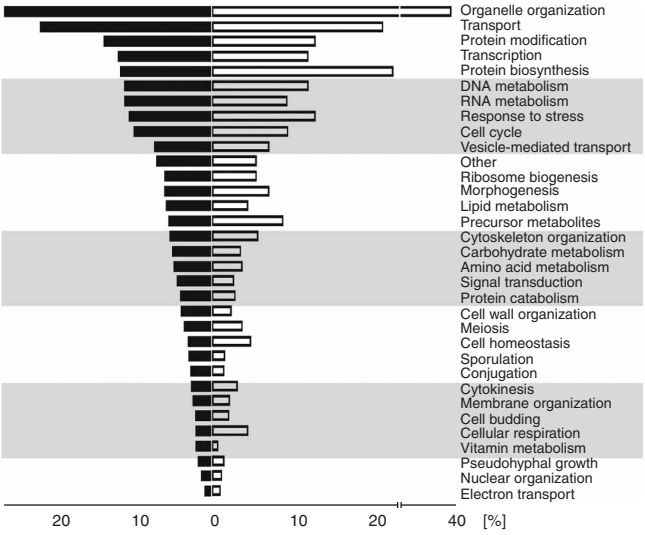

Figure 1 Functional classification according to the 'biological process' annotations in the yeast Gene Ontology slim (see URL in Methods). Percentages of genes annotated with a given category are shown for the whole genome (filled bars) and for the sample used here (open bars). Three categories, organelle organization, transport and protein biosynthesis, are especially abundant in the studied sample. Of these, categories organelle organization and protein biosynthesis are enriched when compared with the genome (hypergeometric test, $P < 1 \times 10^{-10}$ in both cases).

spores that are the direct products of meiosis. Of several such tetrads obtained for each cross, we selected the first in which the following four different genotypes were present: no deletion, either of the two single deletions or both deletions. Every deletion strain was used in two crosses, each time with a different partner. The derived haploid strains were assayed for maximum growth rate. As expected, the single and particularly the double deletion strains showed considerable growth defects (**Fig. 2**). In order to avoid the frequent problem of increased error of estimates for the slow-growing strains, we restricted the measurements to low densities of the assayed cultures when growth was truly exponential (see Methods). The net result was high precision and steady repeatability of estimates across the entire range of growth rates (**Supplementary Fig. 1** online). In principle, the observed growth effects resulted solely from the marked deletions. However, some strains may have harbored unknown mutations that were introduced during the mutagenic process of transformation[15]. Another unwanted by-product accompanying the construction of deletions was aneuploidy, which could have affected an estimated 8% of the collected strains[16]. Linkage between unidentified mutations and markers is unlikely because the budding yeast has 16 chromosomes and about 100 crossovers per meiosis[17]. In a relatively small number of deletion strains, the number or severity of unknown genetic defects must have been exceedingly high. This made tetrad analysis impossible, and therefore the number of testable epistatic effects was smaller than planned (see Methods and sample sizes of statistical tests). Unknown polymorphism could not have affected our results in a systematic way because we deliberately compared strains derived from single tetrads and thus maintained even segregation of potential mutational contamination between the four genotypes.

A population growing continuously at a rate m over time t will grow by the factor e^{mt}, which equals its fitness, w, when t spans exactly one generation[18]. In a model with two loci and two alleles, the absence of epistasis is marked by the equality of multiplicative fitness of the extreme and intermediate genotypes[19] (ww_{kn}, $= w_k w_n$, where k and n stand for the *kan* and *nat* markers). This is equivalent to the additivity of log-fitness, and therefore the epistatic effect can be defined as $\varepsilon = (m + m_{kn}) - (m_k + m_n)$. **Supplementary Table 1** online lists strains used in crosses, their growth rates and calculated values of ε. **Figure 3** presents the frequency distribution of ε. Its mean, median, and modal values are greater than zero and thus show a positive (that is, antagonistic or buffering) role of epistasis. The positive kurtosis of the distribution suggests that there were epistatic effects of especially high value, either positive or negative. However, individual estimates of ε need to be taken cautiously. For example, a negative effect would be spurious if a deleterious, unmarked mutation (not a deletion) segregated into m and m_{kn} while its wild type allele segregated into m_k and m_n. A positive effect would be spurious if the opposite segregation were observed (**Supplementary Fig. 2** online). To learn more about the mean epistasis, $\bar{\varepsilon}$, we investigated its strength in relation to the mean predicted loss in growth rate of the double mutant ($\bar{s}_{k,n} = 2\bar{m} - \bar{m}_k - \bar{m}_n$). The value of $\bar{\varepsilon}/\bar{s}_{k,n}$ was 0.179. As a parallel test of the significance of epistasis, we applied a general linear model analysis. We chose a simple model in which fitness depends on the number of mutations per individual (additive effect) and on the interaction between mutations (epistatic effect), which is proportional to their squared number[20]. As we expected, the growth rate was dependent on the number of mutations (N) and the strength of their fitness effects (C and $C \times N$) (**Table 1**). The epistatic component (N^2) contributed significantly to the variation in growth rate; its effect was not homogeneous but varied between crosses ($C \times N^2$).

We found three synthetically lethal interactions: *vps16Δ* and *atp1Δ*, *gip2Δ* and *pop2Δ*, and *atp15Δ* and *cin4Δ*. These cases of synthetic lethality were not previously documented in the *Saccharomyces* Genome Database (see URL in Methods). Notably, they constitute only 3/642 (0.47%) of all interactions tested here, even though most of our deletion strains carried visible growth defects. A very similar

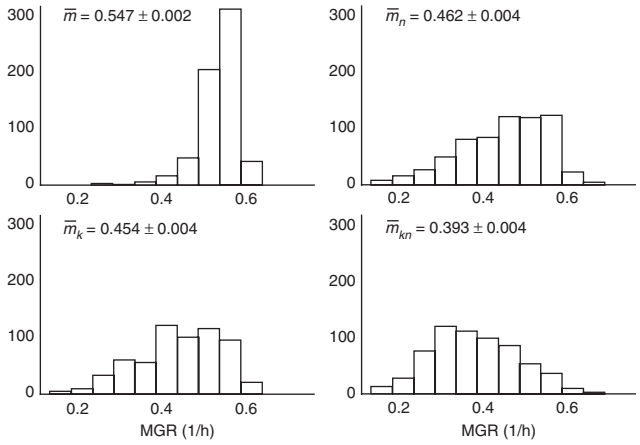

Figure 2 Growth effects of single and double gene deletions. Frequency distributions of the maximum growth rate (MGR) of strains with zero (m), one (m_k and m_n) and two (m_{kn}) gene deletions (mean ± s.e.m. is shown for each category). Kolmogorov-Smirnov tests show that there are differences between the distributions of strains bearing no deletions and strains with a single deletion (m versus m_k: $D = -0.517$, $n_1 = n_2 = 639$, $P < 0.001$; m versus m_n: $D = -0.480$, $n_1 = n_2 = 639$, $P < 0.001$) as well as between those with a single deletion and double deletions (m_k versus m_{kn}: $D = -0.289$, $n_1 = n_2 = 639$, $P < 0.001$; m_n versus m_{kn}: $D = -0.313$, $n_1 = n_2 = 639$, $P < 0.001$) but not between the strains with single deletions (m_k versus m_n: $D = -0.064$, $n_1 = n_2 = 639$, $P > 0.10$).

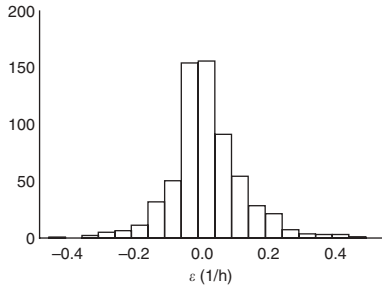

Figure 3 Frequency distribution of the epistatic effect ε. Its mean value, 0.024, is significantly higher than zero ($t = 5.697$, $n = 639$, two-tailed, $P = 1.864 \times 10E{-}08$). The distribution is slightly skewed to the right ($g_1 = 0.282$, $t = 2.917$, $P = 0.0037$) and leptokurtic ($g_2 = 2.803$, $t = 14.518$, $P < 0.0001$).

Table 1 General linear model analysis of the maximum growth rate

Effect	df	MS	F	P
Cross (C)	638	0.05554	12.29	< 0.0001
R(C)[a]	1,788	0.00281	0.62	1.0000
Linear (N)	1	28.62366	6,334.59	< 0.0001
Epistatic (N^2)	1	0.32871	72.75	< 0.0001
C × N	638	0.01960	4.34	< 0.0001
C × N^2	638	0.01104	2.44	< 0.0001
Error	6,003	0.00452		

[a]*R*(*C*) denotes replication within cross.

estimate, 0.44%, was obtained in an earlier study of synthetic lethal interactions that involved deletions of all nonessential genes, many of which were neutral to growth[21]. This suggests that synthetic lethality is a specific, low-frequency phenotype. It is not a generalized threshold trait that is likely to occur when the sum of adverse mutational effects is appropriately high[22].

Our next question was whether particular genes tend to show interactions of defined strength or sign. First, we found that there was not any significant correlation between epistatic effects measured for a single deletion in its two different crosses (Pearson's $r = 0.073$, $t = 1.738$, $n = 565$, $P = 0.083$). Next, we tested whether the cellular role of a gene can influence its average epistatic effect (that is, make it different from the overall mean). We were not able to confirm this for the biological process (**Fig. 4a**), molecular function or cellular localization of the gene product (data not shown). Finally, we asked whether similarity or dissimilarity of the cellular roles of the two interacting genes could be used as a predictor of epistasis and again obtained negative results (**Fig. 4b**). Notably, the functional classes used in these analyses were relatively broad, often comprising hundreds of genes over the whole genome. There were too few pairs of genes from the same or closely related metabolic pathways to allow exploration of physical or direct metabolic interactions between gene products. Close associations between gene products can lead to specific epistatic effects for fitness that differ from the average[10,21]. However, genetic interactions may also arise whenever the cellular response to one mutation modifies the metabolic effect of the next mutation[23]. Most random gene pairs can interact only in this indirect way. Our data show that these abundant interactions, which are likely to contribute to numerous quantitative traits, are mostly moderately positive.

The high prevalence and functional universality of positive epistasis suggests that it may be produced by a common mechanism. In terms of mathematics, the epistasis for fitness, ε, will be positive if the joint effect of multiple deleterious mutations on the rate of growth is not additive but smaller. Metabolic theory predicts that when mutations affect two different enzymes within a single pathway, the resulting maximum rate of metabolic flux (and thus growth) will always be higher than expected by the addition of effects of single mutations[8,9]. The interactions that we have studied mostly concern genes involved in different processes and can be modeled by a metabolic network with independent parallel pathways. **Figure 5** presents two simplified models of this type. In one network, the pathways have a common substrate, and in the other, substrates are different for different pathways. Analysis of both models shows that the epistasis will always be positive (**Fig. 5**). The metabolic network of the yeast cell is far more complex. In recent years, progress has been made at modeling metabolic processes through flux balance analysis (FBA)[24]. One of these studies (ref. 10) specifically addressed interactions between gene deletions in yeast and did not find that either positive or negative epistasis predominated. A likely explanation for this is that the study used deletions with mostly small additive effects and even smaller epistatic ones[10]. In comparison, in our study, we began by selecting strains annotated for slow growth, so small growth effects were underrepresented. However, there was no indication that interactions between deletions with negligible growth effects could be negative. We did not expect any appreciable epistasis between such deletions (**Fig. 5**), nor did we observe any epistasis in crosses involving the least-affected strains (**Supplementary Fig. 2**). To sum up, both our data and theoretical considerations suggest that deleterious mutations

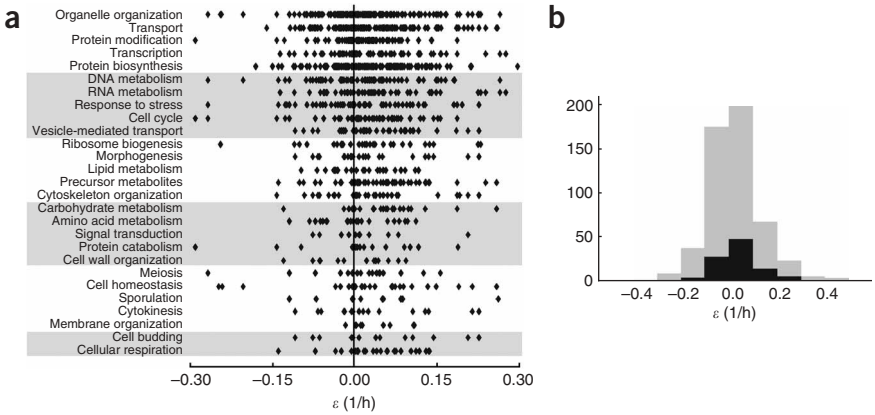

Figure 4 Functional analysis of genetic interactions. (**a**) Univariate analysis of the dependence of the epistatic effect ε on the biological process. The dots represent mean ε calculated from two crosses of each deletion. The Kruskal-Wallis test for homogeneity among groups does not show statistically significant differences ($H_{26,\ 1204} = 20.522$, $P = 0.766$). (**b**) Bivariate analysis. The distribution of ε for pairs of genes that do (black bars) and do not (gray bars) share an annotation of the biological process (yeast Gene Ontology slim, excluding 'process unknown'). The two distributions do not differ statistically (Kolmogorov-Smirnov test, two-tailed, $D = 0.122$, $n_1 = 107$, $n_2 = 532$, $P > 0.10$).

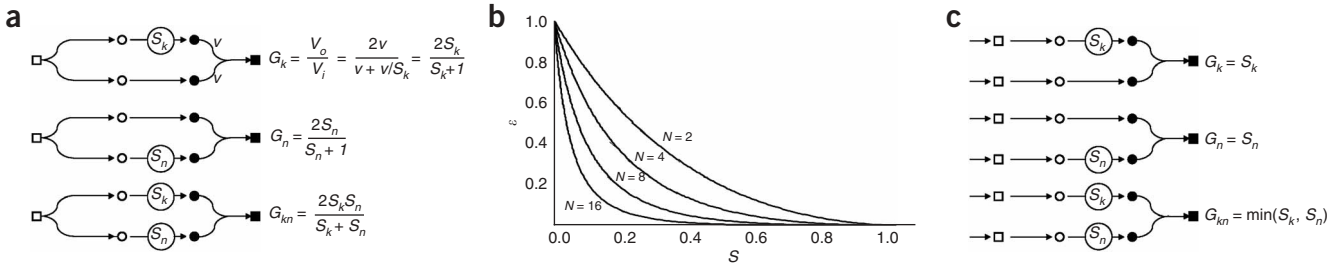

Figure 5 Positive epistasis in simple metabolic networks. Nutrients (empty squares) are converted into intermediate metabolites (empty circles) and then turned into essential components (filled circles) of biomass (filled squares). (**a**) Parallel pathways with a common substrate. Flux balance analysis (FBA) assumes that maximization of output production, V_o, is obtained through optimization of inputs of particular pathways, v, which share an inflowing substrate, V_i. Deletion of a gene (k or n) leads to a reduced effective stoichiometry of reaction, S (with $1 - S$ going to byproducts). Growth G is proportional to efficiency of a network and is normalized to a wild-type genotype ($G_{wt} = 1$). (**b**) In a network with N pathways, the formulas for growth rate of single and double deletion strains become $G_{k,N} = NS_k / ((N-1)S_k + 1)$, $G_{n,N} = NS_n / ((N-1)S_n + 1)$ and $G_{kn,N} = NS_kS_n / ((N-2)S_kS_n + S_k + S_n)$, respectively. The epistatic fitness effect, $\varepsilon = 1 + G_{kn} - G_k - G_n$, will always be positive and highest for networks with few pathways (low values of N) and strong mutational effects (low values of S). (**c**) Parallel pathways with different substrates. Here, growth is limited solely by the slowest pathway. This leads to complete buffering of mutations, with smaller negative effects by mutations with higher negative effects on growth. (The examples above were derived from models presented in Supplementary Fig. 2 in ref. 10).

are likely to reduce the rate of biomass production less than additively on average, which produces positive epistasis. Encouraged by these findings, we suggest that universal determinants of epistasis and genetic robustness can be identified by studying the limitations of biological processes[25] rather than by considering robustness and epistasis as traits optimized by selection[26].

An emerging conclusion is that accumulating deleterious mutations will interact antagonistically if the ability to grow fast is important for fitness, as it is true for the life histories of many organisms. Therefore, our findings are in disagreement with the mutational deterministic hypothesis of the evolution of sexual reproduction, which requires that epistasis be of moderate strength[27], not dominated by opposing strong effects[28] and negative on average[4]. In the present study, the observations of low frequency of synthetic lethality and an average of positive epistasis imply that selection on the mutational load is unlikely to adopt a form of truncation or quasi-truncation. Thus, our data do not suggest that selection would allow accumulation of slightly deleterious mutations up to a critical level beyond which the decline in fitness would have to be abrupt[22].

METHODS

Strains and crosses. We used the haploid deletion strains BY4741 and BY4742 (Open Biosystems YSC1063 and YSC1064). The selected strains were randomly assigned to two groups: *MAT***a**, marked with the *kan*MX4 cassette, providing resistance to geneticin (200 mg/l), and *MAT*α, in which we replaced the former cassette with *nat*MX4, resulting in resistance to nourseothricin (100 mg/l). Pairs of *MAT***a** and *MAT*α strains were arranged at random and mated with each other; two such pairings were done for each strain. The resulting diploid strains were grown for 1 d on GNA agar (peptone 2%, yeast extract 1%, glucose 5%) and then incubated for four days at 25 °C in agitated liquid sporulation medium (1% sodium acetate, 0.005% zinc acetate) with required supplements (5 mg/l uracil, 5 mg/l histidine, 25 mg/l leucine, 7.5 mg/l lysine and 5 mg/l methionine).

Sample size. We found 758 annotations of slow growth. Owing to the absence of some haploid strains in the collection (13 strains) and problems with mating (10 strains), we obtained 735 diploids (two matings of every selected strain would give 758 diploids). Of these, 662 sporulated and germinated efficiently. For most, dissection and examination of four to eight tetrads was sufficient to obtain at least one with the required pattern of marker segregation. For the remaining crosses (735–662 = 73), at least 24 tetrads were dissected. In three crosses, the pattern of synthetic lethality was readily seen, and the remaining 70 showed low efficiency of sporulation and/or germination and no signs of regular marker segregation. This was probably the effect of the aforementioned unknown genetic defects present in the collection. Of the 662 crosses with successful tetrad analysis, 23 cases had to be dropped because they contained strains whose cells tended to clump and form deposits even at relatively low densities of liquid microcultures. In pilot experiments, we found that the use of synthetic medium resulted in considerably more frequent clumping of cells; this was one more reason why we used YPD.

Maximum growth rate estimates. Growth curves were analyzed with Bioscreen C, an automated microbiological system for incubation and measurement of optical density of microcultures. We used a volume of 300 µl of YPD with continuous shaking (low intensity, with a series of six oscillations per cycle), with 20-min intervals between measurements. Growth curves of clearly atypical shape were eliminated before analysis; not only the questioned strain, but all four strains within a tetrad were then re-assayed. To calculate the cell density from the optical density, we used a scaling function that was determined specifically for the microcultures[29]. The lower limit of acceptable readings (the density at which measurements were sufficiently repeatable) was set at 0.1 (corrected for background), and the upper limit (the density at which growth was still exponential) was set at 0.5. The upper limit equaled about one-sixth of the density of the culture after the diauxic shift. With this design, at least eight density measurements fell within these limits. These were log-normal transformed and used to calculate linear regression. Results of pilot experiments (data not shown) demonstrated that the trophic markers and the mating type did not influence the estimates within the applied range of density, nor did we observe any tendency toward nonlinearity of log-transformed data. In effect, the average r^2 (0.9993) was very high. Notably, the average r^2 was nearly uniform along the whole range of growth rates (**Supplementary Fig. 1**).

Earlier experiments demonstrated that the estimates of growth rate done with Bioscreen C showed detectable gradients across microplates[30]. To avoid biases, in this experiment the strains belonging to a single tetrad were always placed in four neighboring wells. Because the assays of growth were replicated four times, the positions of the four compared strains were rotated by 90°. The four replications were started individually; each was used to calculate a single value of ε.

Statistical issues. Two random crosses were done for every deletion. As a result, a single deletion was used to obtain two estimates of ε. The deletion was the same but resided in two independently derived and assayed strains. Moreover, the strain in which the effect of epistasis was likely to occur (that is, the strain with two mutations) was unique for every cross. For these reasons, we regarded every cross as an independent data item. However, we repeated the general linear model analysis for only the first round of pairing. The results, including

the level of type I error, were very similar to those obtained for the whole set of data (**Supplementary Table 2** online). We conclude that performing two rounds of crosses with the same strains could not result in an illegitimate enhancement of statistical power.

URLs. MIPS database: http://mips.gsf.de; *Saccharomyces* Genome Database: http://www.yeastgenome.org.

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

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AUTHOR CONTRIBUTIONS
L.J. performed the experiments and data analyses. R.K. wrote the paper. Both authors planned research and discussed results.

COMPETING INTERESTS STATEMENT
The authors declare no competing financial interests.

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