Functional Characterization of the S. cerevisiae Genome by Gene Deletion and Parallel Analysis

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The functions of many open reading frames (ORFs) identified in genome-sequencing projects are unknown. New, whole-genome approaches are required to systematically determine their function. A total of 6925 Saccharomyces cerevisiae strains were constructed by a high-throughput strategy, each with a precise deletion of one of 2026 ORFs (more than one-third of the ORFs in the genome). Of the deleted ORFs, 17 percent were essential for viability in rich medium. The phenotypes of more than 500 deletion strains were assayed in parallel. Of the deletion strains, 40 percent showed quantitative growth defects in either rich or minimal medium.

The budding yeast S. cerevisiae serves as an important experimental organism for revealing gene function. In addition to carrying out all the basic functions of eukaryotic cells, up to 30% of positionally-cloned genes implicated in human disease have yeast homologs (1). Determining the function of all yeast gene products will be an important step toward understanding their function in metazoans and lays the foundation for a more complete comprehension of cellular processes and pathways.

A powerful way to determine gene function is the phenotypic analysis of mutants missing the gene. Several genome-wide approaches have been proposed including genetic footprinting and random mutagenesis (2, 3). While genetic footprinting has the advantage that all genes can be tested for their contribution to fitness under a particular growth condition relatively quickly, it has the disadvantage that the mutant strains cannot be recovered. In addition, testing each additional condition is as time-consuming as the first. Random mutagenesis is relatively rapid, but the subsequent matching of phenotypes to genes is slower. In addition, with random approaches a certain fraction of genes may be missed, even with oversampling. These limitations can be overcome by deleting each gene in the genome in a directed fashion and by marking each yeast gene with a molecular “barcode” that allows the phenotypes of the mutant strains to be assayed in parallel. The precise deletion of yeast genes can be efficiently accomplished using a polymerase chain reaction (PCR)–mediated gene disruption strategy that exploits the high rate of homologous recombination in yeast (4). For this method, short regions of yeast sequence (≈50 base pairs (bp)) identical to those found upstream and downstream of a targeted gene are placed at each end of a selectable marker gene through PCR. The resulting PCR product, when introduced into yeast cells, can replace the targeted gene by homologous recombination. For most genes, >95% of the resulting yeast transformants carry the correct deletion (5). In addition, this method can be modified so as to introduce two molecular barcodes (UPTAG and DOWNTAG) into the deletion strain. The barcodes or “tags” are unique 20-base oligomer (20-mer) sequences that serve as strain identifiers (6, 7). We show that these barcodes allow large numbers of deletion strains to be pooled and analyzed in parallel in competitive growth assays. This direct, simultaneous, competitive assay of fitness increases the sensitivity, accuracy and speed with which growth defects can be detected relative to conventional methods.

To take full advantage of this approach and to accelerate the pace of progress, an international consortium was organized to generate deletion strains for all annotated yeast genes. Here, we report the construction of precise start-to-stop codon deletion mutants for 2026 ORFs (8).

Genes essential for viability in yeast, in particular those encoding proteins lacking human homologs, have been proposed to be the best targets for antifungal drugs. When spores from the 2026 heterozygous strains were germinated on YPD (yeast extract–peptone–dextrose) media at 30°C haploid deletants could not be recovered for 356 ORFs (see www-sequence.stanford.edu/group/yeast_deletion_project/deletions3.html for an exact list) (9). Despite the considerable interest in these genes as potential drug targets, only 56% of these ORFs had previously been shown to be essential for viability (10). Of the 2026 ORFs analyzed, 1620 were not essential for viability in yeast. For these one additional homozygous and two haploid deletants (Table 1) were also constructed.

A computational Smith-Waterman analysis indicated that 8.5% of the identified nonessential ORFs in the yeast genome have a closely related homolog elsewhere in the genome.
The phenotypic analysis of the deletion strains, in particular those whose cognate protein is not essential to life, is a formidable task. The role of many genes will likely be manifested only under very specialized growth conditions, necessitating the examination of many different conditions. Previous work demonstrated that the barcodes allowed the relative abundance of their respective strains to be measured when 12 strains were grown competitively for many generations (6). The barcoding scheme thus has the potential to accelerate the phenotypic analysis of the deletion strains by allowing the growth rates of all strains to be assayed simultaneously. The first 558 homozygous deletion strains constructed were pooled (12) and grown in rich and minimal media for about 60 generations. During this time, aliquots were removed from the two pools. The tags were amplified, and hybridized to high-density arrays containing the tag complements (Fig. 2A) (13). The hybridization data were used to calculate the relative growth rates for each deletion mutant in the population (14). It was expected that the growth rate for each strain obtained independently with the UPTAG and DOWNTAG signals would agree. For strains in which both the UPTAG and DOWNTAG signal were at least threefold over background, the correlation for growth rates measured with the UPTAG and DOWNTAG were 0.97 in rich medium, and 0.94 in minimal medium (Fig. 2, B and C). The weakest correlations were observed for strains that were the most growth impaired (growth rate <0.6 of that of the wild type) because sufficient signal was detected only for first few time points.

As expected, the strains disappearing at the highest rates in minimal medium but which grew relatively normally in rich medium (at >98% of the pool growth rate) included all of the strains carrying deletions in genes known to be essential for growth in minimal medium including ade1 [tag average <0.50 in minimal (M); 0.98 in rich (R)]; arg5,6 (<0.50, M; 0.98, R); yer068c-a (<0.50, M; 0.99, R; overlaps arg5,6); hil1 (<0.50, M; 1.01, R); ade4 (<0.50, M; 1.0, R); gcn4 (0.53, M; 0.98, R); hom3 (0.54, M; 1.0, R); and ade4 (0.56, M; 1.01, R). In addition, the gypl (0.78, M; 0.99, R) deletion showed a minimal medium-specific growth defect (15). GYP1 (YOR070C) is a GTPase activating protein for Sec4p, a protein in the secretion pathway (16). Mutants of lys7 also showed a growth defect in minimal medium but also grew somewhat slowly in rich medium (0.52, M; 0.88, R). Six strains exhibiting a specific growth defect in rich medium were also identified. These strains included cin8 (0.80 R; 0.95, M); erg6 (0.71, R; 0.96, M); rpl39 (0.85, R; 0.98, M); yml193c-A (0.85, R; 0.98, M; overlaps ribosomal protein rpl36a); esc1 (0.83, R; 0.97, M) and yml013w (0.78, R; 0.95, M).

Altogether, almost 40% of the deletants...
examined showed some sort of growth defect in the competitive growth assay (Fig. 3): 24 (5%) at less than 75% of the pool doubling time; 27 (5%) from 75 to 85%; 80 (15%) at 80 to 98%; and 71 (14%) at 98 to 100%

Strains that grew poorly in rich medium generally also grew poorly in minimal medium aside from the exceptions (e.g. rnr1, hem14) described above. The phenotypic profiles for the deletion strains were in good accordance with those obtained using other methods (17).

It is often assumed that if a gene is expressed under a particular set of conditions, then that gene is important for growth under those conditions. Deletion of the up-regulated gene would then be expected to cause a decrease in growth. These data offered a unique opportunity to correlate changes in gene expression with deletion phenotypes. The tran-

Table 1. Genotypes of strains used in study. For the YD strains, in a few cases deletions were generated in the BY4730 and BY4739 parent strains. These haploid deletants, as well as the resulting homozygous and heterozygous diploids, are HIS+.

<table>
<thead>
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<tbody>
<tr>
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<td>(29)</td>
</tr>
<tr>
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<td>MATa leu2Δ0 lys2Δ0 ura3Δ0</td>
<td>(29)</td>
</tr>
<tr>
<td>BY4741</td>
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<td>(29)</td>
</tr>
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<td>(29)</td>
</tr>
<tr>
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<td>(29)</td>
</tr>
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<tr>
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</tr>
<tr>
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<td>MATa/a orf3::kanMX4/OF3::kanMX4 his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 lys2Δ0/LYS2 met15Δ0/MET15 ura3Δ0/ura3Δ0</td>
<td>This work</td>
</tr>
<tr>
<td>YD30000-YD39999</td>
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<td>This work</td>
</tr>
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Fig. 2. Analysis of 558 homozygous diploid strains in rich and minimal medium. Pools were grown for ~60 generations in minimal medium (SD) supplemented with histidine, uracil, and leucine, or in rich medium (YPD) at 30°C. The batch-transfer method (2, 3) was used to ensure that the cell densities did not exceed 4 x 10^7 cells/ml during the growth study. At least 500,000 cells were transferred during each dilution to avoid unequal representation of strains. Samples (approximately 5 x 10^7 cells) were taken at six different time points for each growth study and genomic DNA was extracted. The two tags for each strain were amplified separately with biotin-labeled primers and were hybridized together to the arrays. (A) Two-color comparison of scanned images of high-density oligonucleotide arrays hybridized with fluorescencelabeled tags amplified from 558 strains grown for 0 (red channel) or 6 hours (green channel) in minimal medium. Approximately 4000 different features, each containing more than 10^7 20-mers of a specific sequence, were synthesized in a specific physical location on the array by photolithography and photosensitive oligonucleotide chemistry (6, 28). Only a portion of the array is shown. The features containing probes to the bar codes for deletion strains that exhibit a growth defect in minimal medium, but not in rich medium, are labeled and indicated with an arrow. The array sequences were assigned sequentially to different deletion strains. A second-generation array has been designed, which contains enough tag complement sequences for every gene in the entire genome.

(B) and (C) Correlation of growth rate data obtained with UPTAG and DOWNTAG sequences for strains grown in rich (B) and minimal (C) media. Data are shown for 331 strains (of the 401 strains that contained both an UPTAG and a DOWNTAG sequence) which had t = 0 UPTAG and DOWNTAG hybridization signals that were both at least threefold over background. More frequent sampling during the initial growth period should improve the correlation. (D) Normalized hybridization intensity data for the 10 slowest-growing (yer014c-a, mtr1, hem14, mot2, pif2, rpl27a, yer044c, rpl50a, ymr118bc, yel1029c) and the 10 fastest-growing strains in rich medium. (E) Hybridization data for the 10 slowest-growing (hem14, arg5, ade1, yer068c-a, ilv1, yer014c-a, yer044c, lys7, gcn4, mtr1) and 10 fastest growing strains in minimal medium.
Deletion map for 336 ORFs and the results of parallel phenotypic analysis for 226 ORFs on chromosome XIII. Data for additional chromosomes can be found at www.sciencemag.org/feature/data/1040380.shl. Chromosome right arms are shown with white backgrounds and left arms with gray. ORFs for which deletions were not generated (gray bars) resulted from failure during PCR or oligonucleotide synthesis (5.2%), failure for unknown reasons (4%), failure to pick unique primers (3.3%), or failure to generate deletions in all four strains (2.5%).

Deletion of genes specifically induced in minimal media was no more likely to affect fitness in minimal medium than deletion of the uninduced genes. Of the genes showing a strong minimal medium growth defect, only ARG4 and ARG5,6 were significantly upregulated (greater than twofold) in minimal medium relative to rich medium. Similarly, only one of the six genes that showed a rich medium–specific growth defect was upregulated more than 2.5-fold in rich medium relative to minimal media. These data indicate the importance of multiple approaches in genome-wide functional analysis studies.

The results we present demonstrate that quantitative fitness data can be rapidly obtained under various conditions. Although the presence of the KanMX4 gene has been shown to have no effect on the fitness of some deletion strains (19), it is theoretically possible that the encoded neomycin phosphotransferase could have an impact on a particular deletion strain. The composition of a pool of deletion strains and the conditions under which the pool was cultured could also have an effect on the observed fitness of the strains. Finally, the phenotypes of certain deletion strains might be complemented by factors released into the medium by other strains in the pool. Additional tests will be required to determine how frequently these artifacts will occur.

These results also show that thousands of deletants can be systematically made once the sequence of a genome is known. Several laboratories in Europe and North America are collaborating to finish construction of tagged deletions for all annotated S. cerevisiae ORFs within 1 year (20). Currently, more than three-quarters of the ORFs in the yeast genome have been deleted (5100 ORFs and more than 15,300 strains generated). Whereas a significant amount of work was expended to construct the strains, in contrast to other methods for generating functional data (2, 3), the strains provide a lasting resource. In addition, the availability of a consistent set of isogenic strains should provide a better way for researchers to compare their results with those of others, easing the task of curating the functional assignments that hitherto have been made in various strain backgrounds. Finally, while other efforts have been mounted by a European consortium and others to generate deletion strains (21), the inclusion of barcodes significantly enhances the usefulness of the strains. The ability to assess thousands of strains quantitatively and in parallel will significantly decrease the amount of labor and materials needed for fitness screens (22) and increase the reliability of the data interpretation and functional classifications.

References and Notes
8. To construct deletion strains, two long oligonucleotide primers are synthesized, each containing (3′ to 5′) 18 or 19 bases of homology to the antibiotic resistance cassette, KanMX4 (U1, D1), a unique 20-bp tag sequence, an 18-bp tag priming site (U2 or D2), and 18 bases of sequence complementary to the region upstream or downstream of the yeast ORF.
were given record numbers between 10,000 and 20,000 (see http://sequence-www.stanford.edu/group/yeast_deletion_project/new_deletion_strategy.html). These 74-mer are to amplify the heterologous KanMX4 module, which contains a constitutive, efficient promoter from a related yeast strain, Asby81, which contains the kanamycin resistance gene, nptII. Because oligonucleotide synthesis is 3' to 5' and the fraction of full-size molecules decreases with increasing length, improved targeting is achieved by reusing the second round of PCR using primers bearing 45 bases of homology to the region upstream and downstream of a particular ORF. Transformation with the PCR product results in replacement of the ORF with the yeast chromosome upon selection for G418 resistance. The unique 20-mer tag sequences are covalently linked to the sequence that targets them to the yeast genome, creating a permanent association and genetic linkage between the particular deletion strain and the tag sequence. The use of two tags increases confidence in the analyses, and the redundancy is useful in case one of the tags carries a mutation or performs poorly in hybridization assays.

To verify correct integration of the deletion cassette, genomic DNA was prepared from the resistant strains and used as template in PCR reactions using two primers, A-KanB and D-KanC, from the KanMX4 module (Figure 1A). PCR reactions were required to give the correct size product when analyzed by gel electrophoresis. If either of the A-KanB or D-KanC reactions failed to yield a product, the identification of the correctly-sized product would fail. In addition, haploid deletion strains were tested for the disappearance of the wild-type AB and CD products. All ORFs encoding proteins greater than 100 amino acids in size were initially selected for deletion. The deletion cassettes were designed to remove the entire coding sequence for a given ORF but to leave the start and stop codon intact. Although ~10% of ORFs in S. cerevisiae overlap one another, the positions of the deletions were not adjusted, nor was any attempt made to avoid essential genes, genes in which a previous deletion had been constructed, or genes with a well-defined function. Genes represented multiple times in the genome (telomeric ORFs, Ty-elements) were usually not chosen for deletion because this would pose a challenge due to the conservation of upstream and downstream regions. Some smaller nonannotated ORFs (NORFs) will be deleted in the future. Transcripts from some of the NORFs have been detected in SAGE analysis, warranting their inclusion in the study (V. E. Velculescu et al., Cell 88, 243 (1997)). All oligonucleotide primers (5 nmol scale) were synthesized on an automated multiplex oligonucleotide synthesizer [D. A. Lashkari, S. Hunick-Smith, R. M. Norgren, R. W. Davis, T. Brennan, Proc. Natl. Acad. Sci. U.S.A. 92, 7912 (1995)] in batches of 96 primers. Scripts were written to automate the selection of primers. Primer sequences and ORF locations were chosen from the Stanford Genome Database (http://genome-www.stanford.edu/Saccharomyces/) at various times over a 2-year period. The KanMX4 cassette was PCR-amplified, and the resulting PCR products were sent to participating laboratories where 1 µg of PCR product was used to transform yeast containing genetic footprinting on the standard laboratory procedure (http://sequence-www.stanford.edu/group/yeast_deletion_project/protocols.html) in a 96-well format. Electronic records, accessible online, were kept for every strain that we constructed. MATa haploid strains were given record numbers of less than 10,000, MATa haploid strains were given record numbers between 10,000 and 20,000, and MATa diploid, between 20,000 and 30,000, and the homogygous diploid, greater than 30,000. Each record consists of primer sequence information, the results of the different diploid tests that were performed, and notes about the phenotype. Data for completed strains are accessible from website sequel. For strains where 1-2 segregation of viability in tetrads was a complete lack of hybridization signal not associated with a mutation in the tag or tagging site. Mutations were most often found in the tags or tag priming sites (0.85% per base in the regions of yeast homology (0.25% per base), most likely due to selection against the mutated PCR products during the recombination event or to the stochastic nature of PCR amplification.

14. Grids were aligned to the scanned images using the known feature dimensions of the array. The hybridization intensities for each of the elements in the grid were determined using the Affymetrix GeneChip software package. Subsequent analysis of the hybridization intensities consisted of two steps: adjustment of data to achieve approximate equality of background and maximal signals on each array, and analysis of the decrease (or increase) of the UPTAG and DOWNTAG signal strength over time. Equalization of signal strength relied on the fact that for most arrays, the amount of tag DNA present did not vary over time. A consensus score for these sites was obtained from the first principal component of the logarithms of the signal intensities. The logarithms were centered and scaled for each element on the array were linearly transformed to make each array's overall signal approximately equal to the consensus. The growth rate for each element on the array was treated as the mean of a model designated as the consensus. All confirmed diploid strains obtained through mating were required to pass two of three tests: a diploid budding pattern, the ability to sporulate, and the inability to mate. In a few cases, essential genes were identified by 2:2 segregation of viability in tetrads. Although a small fraction of signals on each array are liable to have very high values, much larger than can be accounted for by a normal distribution. To determine the existence of a growth defect, deletants were tested in at least three independent biological replicates.

15. The analysis was not as accurate for strains with growth rates of less than 0.5 that of the wild type, because generally only three data points were above background for these.


17. A comprehensive study of chromosome V genes using genetic footprinting (3) provided an opportunity to validate the data: the results generally agreed, with a few exceptions. For example, of the 52 genes whose disruption had no effect on strain fitness under all conditions tested by genetic footprinting, we detected a growth defect in deletants yeo033w (0.68, R; 0.83, M), yeo259c (0.73, R; 0.68, M) and yeo268c (0.79, R; 0.69, M). The observed phenotype for yeo33w probably results from interference with a neighboring gene (HYP2, encoding translation initiation factor eIF-5A). In addition, the hem14 deletion showed a strong genetic defect by itself, while genomic footprinting revealed a salt-specific defect, but not defect in rich medium. Of the 11 genes that had been shown by genomic footprinting to have a severe growth defect, in deletants of genes that carry our deletants, we detected a complete lack of hybridization signal not associated with a mutation in the tag or tagging site. Mutations were most often found in the tags or tag priming sites (0.85% per base in the regions of yeast homology (0.25% per base), most likely due to selection against the mutated PCR products during the recombination event or to the stochastic nature of PCR amplification.
Early Neocortical Regionalization in the Absence of Thalamic Innervation

Emily M. Miyashita-Lin, Robert Hevner, Karen Montzka Wassarman, Salvador Martinez, John L. R. Rubenstein

There is a long-standing controversy regarding the mechanisms that generate the functional subdivisions of the cerebral neocortex. One model proposes that thalamic axonal input specifies these subdivisions; the competing model postulates that patterning mechanisms intrinsic to the dorsal telencephalon generate neocortical regions. Gbx-2 mutant mice, whose thalamic differentiation is disrupted, were investigated. Despite the lack of cortical innervation by thalamic axons, neocortical region–specific gene expression (Cadherin-6, EphA-7, Id-2, and RZR-beta) developed normally. This provides evidence that patterning mechanisms intrinsic to the neocortex specify the basic organization of its functional subdivisions.

The mammalian neocortex is organized into regionally distinct functional subdivisions. There are two proposed mechanisms for neocortical regionalization. The protocortex hypothesis postulates that thalamic afferent fibers play an important role in neocortical regional development (1). On the other hand, the protomap hypothesis (2) postulates that.

20. Contributing groups include all authors. G. Valle, S. Kelley, J. Strathern, and D. Garfinkle.
30. We thank D. Lashkari for establishing the oligonucleotide synthesis facility, T. Nguyen, M. Sigrist, and K. Tanner for help in tetrad analysis, S. Voegele for DNA sequence analyses, P. Koetter for distribution of deletion strains, J. Rine for helpful advice, and M. Cherry and K. Wolfe for files. E.A.W. is supported by the John Wasmuth fellowship in Genomic Analysis (HG00185-02). Supported by NIH grants HG01633, HG01627, HG00198, by an operating grant from the Medical Research Council of Canada, by grants from the European Commission (Bio4-CT97-2294), by the Swiss Federal Office for Education and Science, and by the region de Bruxelles-Capital, Belgium.
62092, USA. The role of the hippocampus in neocortical development is still under investigation.

The hippocampus is one of the many brain regions involved in the regulation of motor coordination and learning. It plays a crucial role in the formation of memory traces, which are fundamental to the process of learning and memory consolidation.

The hippocampus is divided into several regions: the dentate gyrus, the subiculum, the hilar region, and the CA1, CA2, and CA3 subfields. Each of these regions has a distinct function in the regulation of motor coordination and learning.

The dentate gyrus is involved in the formation of new memory traces. It receives input from the entorhinal cortex and projects to the subiculum and CA3 regions. The subiculum is involved in the modulation of the output of the hippocampus, while the CA3 region is involved in the formation of new memory traces.

The CA1 and CA2 regions are involved in the consolidation of memory traces and the formation of long-term memories. The CA3 region is involved in the formation of new memory traces and the CA1 region is involved in the consolidation of memory traces.

The hippocampus is also involved in the regulation of motor coordination. It receives input from the basal ganglia and projects to the cerebellum, which is involved in the regulation of motor coordination.

The hippocampus is a crucial brain region for the regulation of motor coordination and learning. It plays a vital role in the formation of memory traces, which are fundamental to the process of learning and memory consolidation.

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