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[13] Genomic Approaches for Identifying DNA Damage Response Pathways in *S. cerevisiae*

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Abstract

DNA damage response pathways have been studied extensively in the budding yeast *Saccharomyces cerevisiae*, yet new genes with roles in the DNA damage response are still being identified. In this chapter we describe the use of functional genomic approaches in the identification of DNA damage response genes and pathways. These techniques take advantage of the *S. cerevisiae* gene deletion mutant collection, either as an ordered array or as a pool, and can be automated for high throughput.

Introduction

Genetic studies in the budding yeast *Saccharomyces cerevisiae* have uncovered many components required for proper and efficient execution of the cellular response to DNA damage. Recent advances in genomic, proteomic, and bioinformatic techniques have further increased the utility of *S. cerevisiae* as a model organism for the study of the DNA damage response. In particular, the construction of a complete collection of *S. cerevisiae* gene deletion mutants (Giaever *et al.*, 2002; Winzeler *et al.*, 1999), along with libraries of conditional alleles of essential genes (Kanemaki *et al.*, 2003; Mnaimneh *et al.*, 2004), have allowed for systematic genetic analyses to determine gene function. Although the techniques described in this chapter make use of the haploid gene deletion collection, in principle they can be applied to any

collection of mutants, with the exception of the barcode-based screening approaches.

Systematic Genome-Wide Screens to Identify Genes Required for Resistance to DNA Damaging Agents

Array-Based Screening

Array-based approaches have been successfully employed to screen for mutants that are sensitive to ionizing radiation (Bennett *et al.*, 2001), methyl methanesulfonate (Begley *et al.*, 2002; Chang *et al.*, 2002), wortmannin (Zewail *et al.*, 2003), bleomycin (Aouida *et al.*, 2004), hydroxyurea, camptothecin (Parsons *et al.*, 2004), oxidative stress (Tucker and Fields, 2004), and etoposide (Baldwin *et al.*, 2005). These screens have typically used the *S. cerevisiae* gene deletion collection, a collection of some 4700 strains, each carrying a deletion of a single nonessential open reading frame (Giaever *et al.*, 2002; Winzeler *et al.*, 1999). This collection has been screened both as haploids (Aouida *et al.*, 2004; Baldwin *et al.*, 2005; Chang *et al.*, 2002; Parsons *et al.*, 2004; Tucker and Fields, 2004; Zewail *et al.*, 2003) and as homozygous diploids (Bennett *et al.*, 2001). The gene deletion collection is available from Open Biosystems (Huntsville, AL; <http://www.openbiosystems.com/>), EUROSCARF (<http://web.uni-frankfurt.de/fb15/mikro/euroscarf/complete.html>), and the ATCC (<http://www.atcc.org/common/specialCollections/cydac.cfm>), and is supplied as frozen cell suspensions in 96-well plates or as 96 strains stamped on agar plates.

Screens with the gene deletion collection are facilitated by the construction of an ordered array of the mutant strains such that the identity of the mutant at each address on the array is known. This allows for the rapid identification of mutants detected in a given screen. We use an array constructed on 86 mm × 128 mm agar plates formed in single well Omni-Trays (Nunc, Rochester, NY), at a density of 768 colonies per plate. With each strain present in duplicate, this array occupies 16 plates. This array was originally designed for use in synthetic genetic interaction screens (Tong *et al.*, 2001), and the construction of such an array, using the gene deletion collection as supplied in 96-well plates by Open Biosystems, has recently been described in detail (Tong and Boone, 2006). A density of 768 colonies per plate provides a reasonable balance between high density and the ability to reproducibly transfer cells from plate to plate using a replica pinning procedure, but is more suitable for replica pinning using a robotic system. In practice, a lower density, such as 384 colonies per plate, will be easier to work with if the replica pinning is done by hand rather than by a robot. This, of course, necessitates a larger array of some 32 plates. At the

other extreme, the use of a contact microarrayer to print and manipulate very high density arrays of up to 9600 colonies per OmniTray has been described, with the advantage that considerably smaller amounts of drug are required to screen the deletion collection in this format (Xie *et al.*, 2005).

Using an ordered array of the gene deletion collection, genome-wide screening to identify genes required for resistance to DNA damaging agents is performed by transferring the yeast strains from the master array onto solid medium containing the given drug and to the same medium lacking the drug. The concentration of drug to be used in a screen can be determined by several approaches. If known drug sensitive mutants are available, a serial-dilution spot assay, described later, can be performed to compare sensitivity to a wild-type strain at several different drug concentrations. If suitable positive controls are not available, a concentration of drug that causes a mild inhibition of growth of the wild-type strain can be used. In either case, pilot studies can be performed with one or several gene deletion collection array plates to test sensitivity at several drug concentrations across several hundred mutant strains. Plates chosen should have several reasonable candidates for sensitive strains, for example, mutants in genes in known DNA-damage response pathways.

The transfer of colonies to the solid medium containing the drug is typically accomplished by a replica pinning procedure, performed manually or with a robot. The sensitivity of each of the strains to a particular drug is assessed by comparing colony size on the plate containing the drug to the size on the no-drug control plate. Since each strain is present twice on the array, the duplicates provide an internal control for variation in pinning. A strain is only scored as positive in a screen if both colonies are small or absent. We perform each screen against the complete array at least three times. Identification of sensitivity in at least two screens serves as a convenient threshold for further confirmation using serial-dilution spot assays, described later. Computational methods for scoring colony size differences have also been described (Tong *et al.*, 2004). One obvious drawback of array-based screening methods is that they consume a lot of drug. In cases where the drug is limiting, competitive growth screening methods, described later, are more suitable. As with any screening procedure, these screens are subject to both false-positives and false-negatives. False-positives (which typically fall in the 11–22% range) are eliminated by confirming the sensitivity of mutants identified in the primary screen by an independent secondary screen (Aouida *et al.*, 2004; Chang *et al.*, 2002). We typically use a serial-dilution assay, again on solid media, as described later. In principle, confirmations could also be performed as growth assays in liquid culture, although without automation the serial-dilution assay has higher

throughput. False-negative rates can be estimated by comparing the mutants identified in the primary screen with those mutants known to confer sensitivity to the given drug. Although this would not be possible for a new drug, when this analysis was applied following screens for MMS and bleomycin sensitivity, estimates of 44% and 26% false negatives were obtained (Aouida *et al.*, 2004; Chang *et al.*, 2002).

Manual Replica Pinning

Manual pinning tools can be purchased from V & P Scientific, Inc. (San Diego, CA; <http://www.vp-scientific.com/>) in both 96- and 384-floating pin formats. Alignment manifolds are also available from V & P Scientific for use in array construction and for precise alignment of array replicas on OmniTray plates. Plastic tip box lids or OmniTrays are used as reservoirs for cleaning and sterilizing the pinning tools.

Pinning Procedure

1. Sterilization of manual pinning tools:
 - a. Set up 8 wash reservoirs as follows:
 - i. Water reservoirs: 1, 2, 3, 4, 6, 7
 - ii. 10% bleach: 5
 - iii. 95% ethanol: 8
 - b. Move manual pinning tool from reservoirs 1 through 8. Let pinning tool sit in first two water reservoirs for at least ~1 min each, with gentle shaking, to remove cells on the pins. Change first 2 water reservoirs periodically (i.e., every 2 or 3 rounds of washing).
 - c. After wash in last (i.e., 95% ethanol) reservoir, allow excess ethanol to drip off the pins, then flame the pins. Allow pinning tool to cool.
2. After sterilizing the pins, replica-pin the mutant strains from the master array onto agar plates containing the selected DNA damaging agent. Make a second replica onto agar plates lacking drug as a control. The two replicas can be made from a single pinning off of the master.
3. Incubate plates at 30° for 1–3 days. Compare growth of colonies on plates containing drug with the growth on plates lacking drug. Score colonies where both of the duplicates show reduced or no growth on the plate that contains the drug. An example of a replica pinning experiment at 768-colony density is shown in Fig. 1.

More sophisticated pinning protocols where multiple replicas are made without returning to the master plate have been described (Aouida *et al.*,

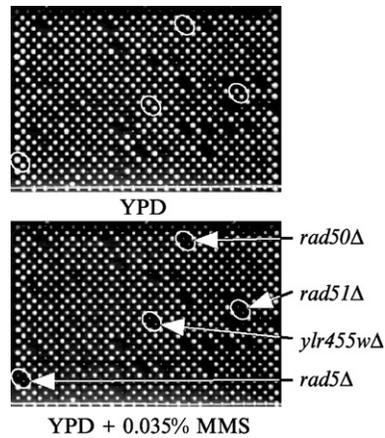


FIG. 1. A screen for methyl methanesulfonate sensitivity using replica pinning. The complete set of haploid yeast deletion mutants was arrayed in duplicate onto 16 plates and pinned onto YPD media or YPD + 0.035% MMS (array plate 11 of 16 is shown). Putative MMS sensitive mutants lead to the formation of smaller colonies when grown on MMS-containing media. Reproduced from [Chang *et al.*, 2002](#), copyright 2002, National Academy of Sciences, USA.

2004). These can produce a dilution effect and reveal sensitivities that are not evident in a single pinning. Although the previous method uses the gene deletion collection of nonessential genes, it could in principle be adapted to screen essential genes. This could be done using heterozygous mutant strains carrying deletions of essential genes to screen for haplo-insufficiency in the presence of drug. Additionally, a collection of strains carrying essential open reading frames under the control of a tetracycline-regulated promoter has been described ([Mnaimneh *et al.*, 2004](#)). This collection could be screened for drug sensitivity in the presence of different concentrations of the tetracycline analogue doxycycline in order to reduce expression of essential genes.

Robotic Pinning Systems

There are several robotic systems available for replica pinning. These include the VersArray colony arrayer system (BioRad Laboratories, Hercules, CA), the QBot, QPixXT, and MegaPix (Genetix, Boston, MA), and the Singer Rotor HDA bench top robot (Singer Instruments, Somerset, UK). These systems provide greater precision, reproducibility, and throughput than hand pinning, but at increased expense.

Confirmation of Sensitivity Using Serial-Dilution Spot Assays

1. Grow deletion strains overnight to saturation at 30° in 2 ml yeast extract-peptone-dextrose (YPD).
2. Dilute cultures using sterile water to a concentration of 10⁷ cells/ml (or approximately OD₆₀₀ = 0.5).
3. Using a multichannel pipettor and a 96-well microtiter plate, further dilute each culture by making 4 serial 10-fold dilutions.
4. Spot 5 μl of each dilution onto medium containing drug and medium lacking drug. Using agar plates formed in single-well OmniTrays, it is possible to analyze 4 dilutions of 12 strains per plate.
5. Incubate plates at 30° for 2–4 days to allow for cell growth before scoring for drug sensitivity. An example of a spot assay is shown in Fig. 2.

Barcode-Based Screening

Because many compounds are in limited supply, a major challenge is to screen the highest number of mutants in the most efficient manner possible while using the least amount of growth medium. Parallel analysis of large numbers of pooled deletion strains in a minimal amount of media is possible due to the two unique molecular barcodes (“uptag” and “downtag”) that tag and identify each deletion strain (Shoemaker *et al.*, 1996; Winzeler *et al.*, 1999). This method was originally developed to identify drug targets through

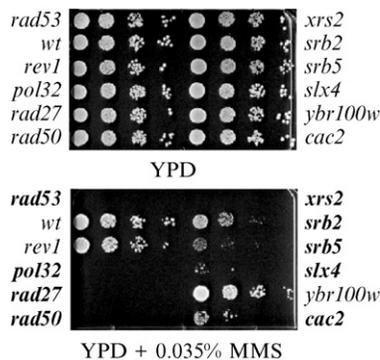


FIG. 2. Confirmation of MMS sensitivity. Putative MMS sensitive strains were grown in YPD overnight at 30°. Serial 10-fold dilutions were spotted onto YPD or YPD + 0.035% MMS and incubated at 30° for 3 days. Strains in **bold** were scored as sensitive. A *rad53* mutant was used as a positive control. Reproduced from Chang *et al.*, 2002, copyright 2002, National Academy of Sciences, USA.

screens of the yeast heterozygote deletion collection for drug-induced haplo-insufficiency (Giaever *et al.*, 1999, 2004; Lum *et al.*, 2004) but it has since been modified for many different applications. In this strategy, presented in schematic form in Fig. 3, strains are pooled and grown in parallel in liquid culture under selective conditions (for example, in the presence of a drug compound). Genomic DNA is extracted from the pool and the barcodes from each strain in the pool are simultaneously amplified using common flanking primers fluorescently labeled with Cy3 and Cy5. This pool of PCR amplimers is then hybridized to high density DNA microarrays

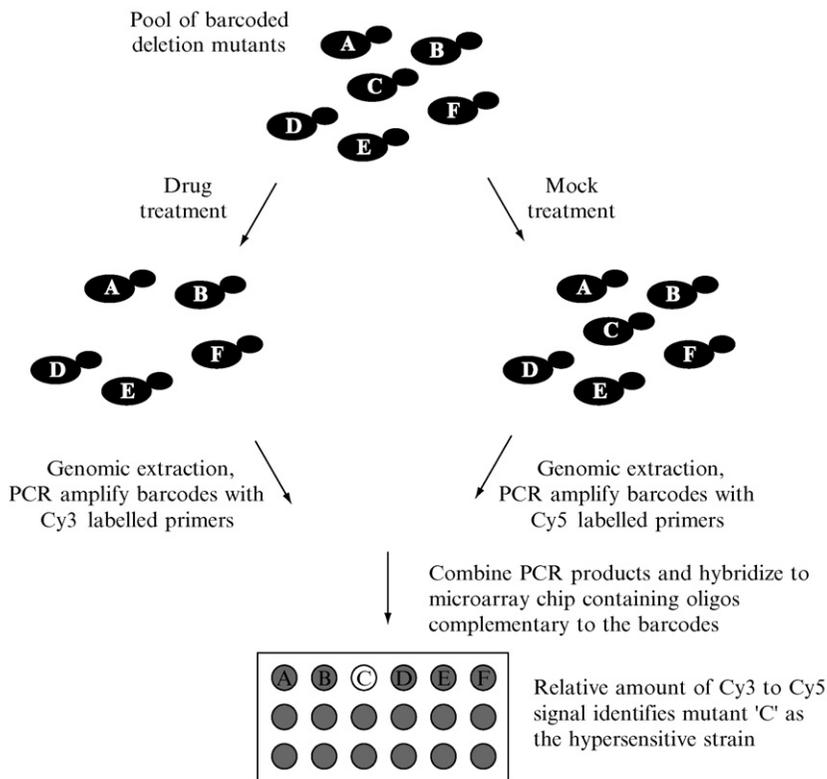


FIG. 3. Parallel analysis of large pools of deletion mutants. Populations of pooled mutant cells each marked with unique molecular barcodes are grown in the presence or absence of a growth inhibitory drug. Genomic DNA is extracted from the pool of mutants and barcodes representing each strain are amplified by PCR using common primers labeled with the fluorescent markers Cy3 or Cy5. Drug sensitive mutants are identified by competitive hybridization of the barcode PCR products to a microarray containing oligos corresponding to each barcode.

containing oligonucleotides corresponding to the barcodes. The relative abundance of each strain in the pool is assessed by the strength of the resulting signals from the microarray readout compared to a mock control. Using this approach, the entire yeast deletion set can be pooled, grown competitively, and quantitatively assessed in small volumes of growth medium (Giaever *et al.*, 2002).

A number of groups have used this strategy to screen the yeast deletion mutant collection for drug hypersensitivity to uncover the pathways and cellular functions affected by drug treatment on a global scale. Giaever and colleagues profiled the nearly complete set of *S. cerevisiae* deletion strains under a number of different conditions including high salt, sorbitol, galactose, pH 8, minimal media, and nystatin treatment (Giaever *et al.*, 2002). Other groups have used this method to screen DNA damaging agents. For example, Hanway and colleagues analyzed a collection of 2827 homozygous non-essential deletion strains for UV and MMS sensitivity and implicated six genes not previously known to be involved in DNA damage repair pathways in the DNA damage response (Hanway *et al.*, 2002). A separate group also screened the deletion mutant collection for strains hypersensitive to UV radiation (Birrell *et al.*, 2001). This study identified three novel genes that lead to UV sensitivity when deleted, two of which have human orthologs associated with cancer. A screen for sensitivity to ionizing radiation yielded a novel radiation resistance gene, *RAD61* (Game *et al.*, 2003). The anticancer agents cisplatin, oxaliplatin, and mitomycin C have also been used in screens (Wu *et al.*, 2004).

While difficult to determine for certain, the overlap between yeast cell array based chemical screens and barcode based screening appears to be around 30–60% (Parsons and Boone, unpublished results). Discrepancies between the array-based and barcode screens are likely a result of intrinsic differences in the assays. The array-based approach is performed on solid media with each strain pinned individually onto medium and allowed to grow without significant competition from other strains. In contrast, chemical-genetic profiling is a competitive fitness assay performed in liquid media. Often, minimum inhibitory drug concentrations vary widely in liquid medium compared to solid medium. In addition, the barcodes are known to carry some mutations; however, they have now been sequenced (Eason *et al.*, 2004) and so new arrays should lead to more accurate profiles.

Protocol for Barcode-Based Chemical-Genetic Screening

1. Pool yeast strains and freeze down in aliquots.
 - a. Using a 96-pin hand pinner, pin strains from 96-well stock plates onto YPD agar plates

- b. Grow for 2 days at 30°
- c. Scrape cells off plates into YPD + 15% (v/v) glycerol
- d. Adjust pool to a cell density between $4 \times 10^8 - 8 \times 10^8$ cells/ml
- e. Freeze at -80° in 1 ml aliquots.

2. Thaw pool and dilute to a final concentration of 5×10^5 cells/ml in 5 ml of YPD. If the volume of drug is limiting, it is possible to use smaller volumes of media (less than 1 ml).

3. Expose pool to drug at semi-inhibitory concentration where the growth of the pool is inhibited by approximately 20–30%. Also include a mock-treated solvent control.

4. Incubate at 30°, with shaking, for 14 h

5. Dilute strains to 5×10^5 cells/ml and add fresh compound or solvent to the tubes. Incubate at 30°, with shaking, for 24 h.

6. Prepare genomic DNA from 1.5 ml of culture using yeast DNA extraction kit (MasterPure™ Yeast DNA Purification Kit; Epicentre Cat # MPY80200, Madison, WI) and normalize DNA concentrations to 25 ng/ml.

7. PCR amplify barcodes using 5' Cy3 and Cy5 labeled common primers. Amplify the drug treated pool with one fluor and the control pool with the other fluor. Primers U1 (GATGTCCACGAGGTCTCT), and U2 (CGTAC-GCTGCAGGTCGAC) are used to amplify the uptags and D1 (CGGTGTC-GGTCTCGTAG) and D2 (ATCGATGAA TTCGAGCT CG) to amplify the downtags. Amplify the uptags and downtags in separate reactions.

PCR reactions:

4 μ l gDNA (25 ng/ml)

2 μ l Cy3/5-U1 or Cy3/5-D1 (16.5pM/ μ l)

2 μ l U2 or D2 (16.5pM/ μ l)

67.5 μ l Platinum PCR Supermix (Invitrogen; Cat # 11306-016, Grand Island, NY)

94° for 2 min

35 cycles of:

94° for 30 sec

50° for 30 sec

72° for 30 sec

Then 72° for 5 min

4° for ∞ .

8. Combine PCR products (uptags and downtags from the drug-treated pool and uptags and downtags for the mock-treated pool) and hybridize to an oligonucleotide chip (we use Agilent custom microarrays) containing oligos complementary to the barcode sequences. All barcode and primer sequences are available at http://www-sequence.stanford.edu/group/yeast_deletion_project/. Barcode oligos can be purchased from Invitrogen (Cat # 40904).

9. Hybridization procedure:
 - a. Combine 65 μl of PCR product from each of 4 reactions:
 - i. Cy3 labeled uptags from drug-treated pool
 - ii. Cy3 labeled downtags from drug-treated pool
 - iii. Cy5 labeled uptags from mock-treated pool
 - iv. Cy5 labeled downtags from mock-treated pool. Also run a fluor-reversed chip with the drug-treated pool labeled with Cy5 and the mock treated pool labeled with Cy3.
 - b. Add 10 μl each blocking mix (up blocking mix: 25 pM/ μl each of U1 and U2-RC [GTCGACCTGCAGCGTACG]; down blocking mix: 25 pM/ μl each of D1 and D2-RC [CGAGCTCGAATCATCGAT]). These blocking mixes are complementary to the common priming sites of the uptags and downtags, respectively, and are designed to reduce nonspecific hybridization.
 - c. Boil at 100° for 4 min, then cool on ice for 5 min.
 - d. Add 170 μl hybridization buffer (for 5 ml: 2650 μl 5 M NaCl, 132.5 μl Tris-HCl pH 7.5, 1325 μl 5% Triton X-100, 882.5 μl H₂O, 10 μl 1 M DTT).
 - e. Add entire mixture to an Agilent hybridization chamber (Agilent; Cat # G2534A).
 - f. Hybridize at 42° for 4 h with slow rotation.
 - g. Wash chips by dipping 5 times in 6X SSPE/0.05% TritonX100, then 5 times in 0.06X SSPE at 4°.
 - h. Scan chips immediately. We use a GenePix 4000B scanner from Axon instruments (Sunnyvale, CA) to scan the chips and Array-Pro Analyzer from MediaCybernetics (Silver Spring, MD) to analyze the images.

Synthetic Genetic Array Analysis to Identify DNA Damage Response Pathways

Of the ~6000 known or predicted genes in *S. cerevisiae*, about 75% are nonessential (Giaever *et al.*, 2002; Tong *et al.*, 2004; Winzeler *et al.*, 1999). This emphasizes the ability of yeast cells to tolerate individual deletions of most genes, likely reflecting redundant pathways that have evolved to buffer the phenotypic consequences of genetic variation (Hartman *et al.*, 2001). This high degree of genetic redundancy makes it difficult to determine the function of many genes, but studying synthetic genetic interactions can circumvent this problem. A synthetic genetic interaction occurs when a mutation in a gene suppresses, enhances, or modifies the phenotype of a second mutation. In particular, if these two mutations cause cell sickness or cell death, the synthetic genetic interaction is termed synthetic sick or synthetic lethal, respectively. The creation of the *S. cerevisiae* gene

deletion mutants has enabled genome-wide, high-throughput synthetic genetic interaction screens using an approach termed synthetic genetic array (SGA) analysis (Tong *et al.*, 2001, 2004). Large-scale SGA analyses have proven to be very useful for predicting gene function because genetic interactions often occur between functionally related genes, and similar genetic interaction profiles tend to identify components of the same pathway (Tong *et al.*, 2004). SGA screens with known DNA-damage response genes successfully identified *ELG1* as a gene important for DNA replication and genomic integrity (Bellaoui *et al.*, 2003), and helped reveal sister chromatid cohesion roles for the DNA damage response MRX complex (Warren *et al.*, 2004) and the S-phase checkpoint genes *TOF1* and *CSM3* (Mayer *et al.*, 2004).

SGA Methodology

In a typical SGA analysis, a query strain containing a mutation in the gene of interest is crossed to each arrayed deletion mutant. The resulting doubly heterozygous diploids are replica-pinned onto sporulation media to yield haploid progeny that have undergone meiotic recombination, which are subsequently replica-pinned onto media that only allow growth of haploid yeast containing both the query mutation and deletion array mutation. Growth rates of the double mutants are assessed to identify synthetic genetic interactions. A detailed protocol to carry out the SGA methodology has recently been described (Tong and Boone, 2006). A schematic diagram of the SGA procedure is presented in Fig. 4.

Because fitness defects of double mutants can also be examined using the barcode approach with a microarray-based readout as described previously for chemical genetic screens, SGA analysis can also be carried out using a pooled population of deletion mutants. In addition, rather than crossing a mutation of interest into the set of ~5000 viable deletion mutants, the query mutation can be introduced by transformation into heterozygous diploids containing the SGA reporter, a method referred to as dSLAM (Pan *et al.*, 2004).

Clustering Analysis

In addition to performing SGA screens with known DNA damage response mutants, the analysis of the genetic interaction data from the greater than 130 SGA screens performed to date has proven to be a useful strategy for the identification of genes involved in the response to DNA damage. Clustering analysis is a useful tool for analyzing quantitative data, and has been used to visualize and analyze the large data sets from multiple SGA screens (Tong *et al.*, 2004). It can be used to find patterns of similar

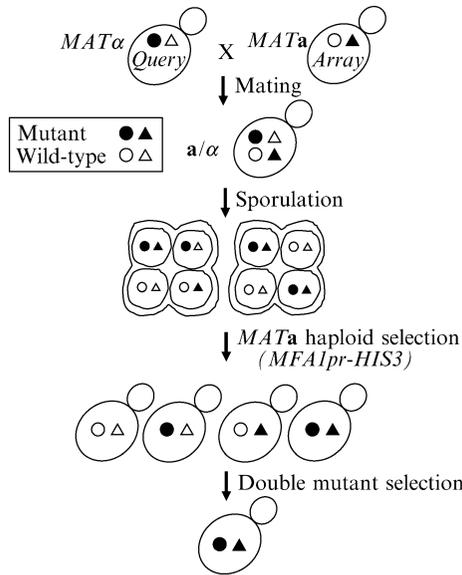


FIG. 4. Synthetic genetic array (SGA) analysis. The query mutant strain is crossed to the ~4600 strains that make up the viable haploid deletion mutant collection. Since the query and the deletion mutants are marked with different dominant selectable marker, diploids can be selected by replica pinning to appropriate media. Diploids are induced to sporulate by pinning to sporulation medium. Spores are germinated on medium that selects for *MAT α* haploids, and double mutants are selected from among the haploids. The fitness of the double mutants is scored by comparing their growth to a control cross in which the deletion mutant array is crossed to a wild type strain.

behavior in a set of Items (i.e., genes) in relation to a set of Criteria (i.e., experiments). When clustering, the data is represented in a matrix with Items along one axis, Criteria along the other, and an Item's measurement for a given Criterion at their intersecting coordinate. The aim of clustering is to reorganize the matrix such that Items behaving most similarly along the Criteria axis are next to each other, and Criteria behaving most similarly along the Items' axis are next to each other. In terms of the data discussed here, the "Items" will be deletion strains and the "Criteria" are the different screens, either genetic or chemical. The difference between two Item or Criterion vectors is calculated based on a distance metric. Different metrics may be available in different clustering applications. Users should refer to the documentation of the application they are using to see what metrics are available to them.

In the case of SGA screens, this analysis groups mutants on the basis of the similarity of their genetic interaction profiles. Thus if two deletion

mutants display synthetic genetic interactions with the same deletion mutants in a genome-wide analysis, they will likely cluster next to each other. Of particular importance, genes whose products function in the same pathway, biological process, or protein complex frequently cluster together (Tong *et al.*, 2004). Because of this tendency, clustering analysis has predictive value in identifying new genes that function in a given process, and in assigning functions to genes with unknown cellular roles. Clustering analysis helped place *CSM3* in an S-phase checkpoint pathway involving *MRC1* and *TOF1* (Tong *et al.*, 2004), and *RMII* in a genomic integrity pathway with *SGS1* and *TOP3* (Chang *et al.*, 2005). The relevant portion of a clustering analysis that indicates that *CSM3* has a genetic interaction profile similar to that of *MRC1* and *TOF1*, and that *RMII* has a genetic interaction profile similar to that of *SGS1* and *TOP3* is presented in Fig. 5.

Cluster 3.0 and Java Treeview

Cluster 3.0 and Java Treeview provide a simple and basic way to perform clustering analysis and view its results. Further, being open source, they are widely accessible at no cost, and there is room for customization and contribution. Cluster 3.0 is an Open Source Clustering Software resource, provided by Michiel de Hoon at the University of Tokyo, with clustering tools available for Windows, Linux, Unix, and Mac OSX and is located at: <http://bonsai.ims.u-tokyo.ac.jp/~mdehoon/software/cluster/>. Java Treeview (<http://genetics.stanford.edu/~alok/TreeView/>) is an application developed by Alok Saldanha that is useful for visualizing the hierarchical clustering results.

How to Use Cluster 3.0 and Java Treeview

Cluster 3.0 has a graphical user interface that can be used to load, analyze, and save data. Cluster 3.0 can open tab-delimited data files. The data file must be laid out in a matrix format, where the rows represent Items and columns represent Criteria. (For example, see Table I.)

The first column contains the Item name or label for each gene, and the first row contains the label for each Criterion. The first field of the first row describes what Items are represented along the rows. Each data cell contains the measurement for its respective Item and Criterion. More advanced formatting allows for the assignment of weights to each Item and Criterion. Please refer to the online documentation at <http://bonsai.ims.u-tokyo.ac.jp/~mdehoon/software/cluster/manual/index.html> for details.

There are several tabs in the Cluster 3.0 interface that allow various tasks to be performed once data is loaded. The “Filter Data” and “Adjust Data” tabs allow filtration and normalization in preparation for clustering,

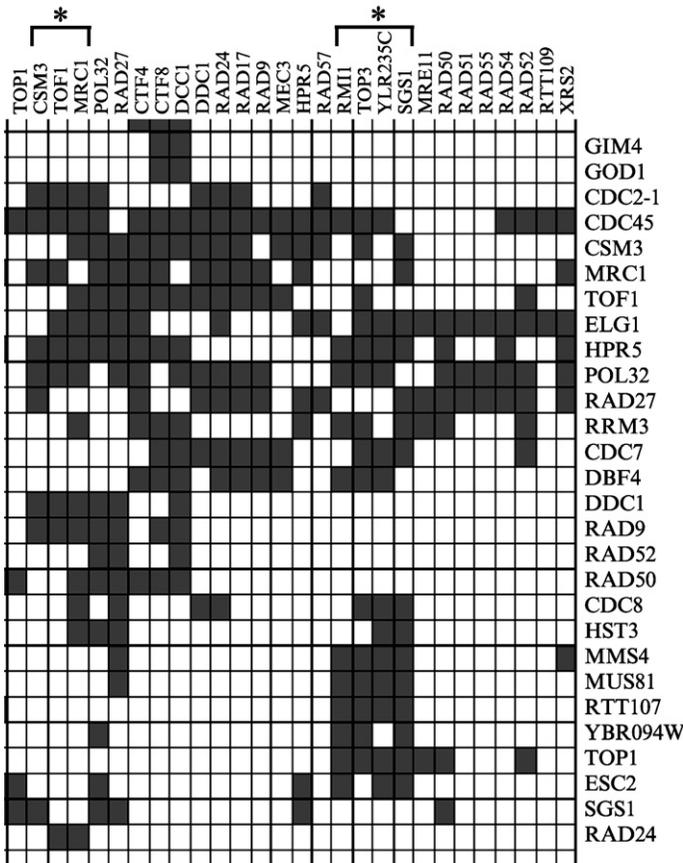


FIG. 5. Clustering analysis can predict gene function. A section of a clustering analysis of synthetic genetic interactions determined by SGA analysis is presented. The rows are the query genes and the columns are the deletion mutant array genes. Synthetic genetic interactions between two genes are represented as dark squares at the intersection of the query gene row and the array gene column. The clusters containing *CSM3*, *MRC1*, and *TOF1*, and *RMI1*, *SGS1*, and *TOP3* are indicated by “*”.

although this is normally not required for SGA or chemical data. The remaining tabs allow Hierarchical, K-means, and SOM clustering and Principle Component Analysis, respectively. Hierarchical clustering is used most commonly with SGA data. Here, pairs of Items are linked together into clusters based on decreasing similarity. A tree of linkages is built from the bottom up, starting with a cluster containing a pair of very similar Items in a single cluster up to an umbrella cluster containing loosely linked

TABLE I
 EXAMPLE OF A DATA FILE FORMATTED FOR CLUSTERING ANALYSIS IN CLUSTER 3.0.
 IN THIS TABLE OF DNA DAMAGING AGENT SCREENS A "1" REPRESENTS
 SENSITIVITY AND A "0" REPRESENTS NO SENSITIVITY. IN A SIMILAR ANALYSIS OF SGA
 DATA, THE VALUES WOULD BE "1" FOR A SYNTHETIC LETHAL OR SYNTHETIC SICK
 GENETIC INTERACTION AND "0" FOR NO GENETIC INTERACTION

Gene	HU	CPT	MMS	UV	IR
AAT2	1	0	1	0	0
ADH1	1	1	0	0	0
ADK1	0	0	0	0	1
ADO1	0	0	0	0	1
AKR1	1	1	0	0	1
ANC1	0	0	1	0	1
AOR1	0	0	1	0	0
APN1	0	0	1	0	1
ARD1	0	0	0	0	1
ARO1	0	0	1	0	0
ARO2	1	0	0	0	0
ARO7	0	0	1	0	0
ARV1	0	0	0	0	1
ASF1	1	1	1	1	1

subclusters. This method of clustering gives highly reproducible results and provides trees that describe the hierarchy of clusters based on similarity.

The clustering tabs allow users to select their clustering parameters and metrics. There are several distance metrics available in Cluster 3.0, which include Centered and Uncentered Correlation, Spearman Rank, Kendal Tau, and Euclidean Distance. Distance metrics are used to determine the similarity between two Items or clusters: the less the distance the greater the similarity. The most commonly used similarity metric is based on the Pearson correlation, and this can be applied to the data by choosing Correlation (centered) in the Similarity Metric dialog box. Details on this and the other distance metrics may be found in the Cluster 3.0 online documentation (<http://bioinfo.tau.ac.il/man/cluster/html/Distance.html>).

Once clustering analysis is executed, Cluster 3.0 automatically generates result files for use with Java Treeview (Warning: result files are generated using the prefix of the input file followed by ".", then the suffix associated with the clustering method. These files are placed in the same directory as the input file. Any pre-existing file with the same name as a result file will automatically be overwritten). After the clustering analysis is completed, the user may save the updated data file.

Once the result files have been generated they may be viewed using Java Treeview. Java Treeview can open result files with a ".cdt" or ".pcl"

suffix. The interface consists of several frames. When loading a file, Java Treeview displays the result matrix in the main frame, as well as trees describing relationships in the case of hierarchical clustering. The user may select a region of the result matrix using the mouse or by selecting tree branches and view information on particular spots. The user can set various color schemes, perform analysis, and retrieve basic statistics. The user may also save the matrix data and export the matrix image, trees, and labels to a Postscript or Image file. An example of a clustering analysis using data from screens with methyl methanesulfonate (Chang *et al.*, 2002), hydroxyurea (Parsons *et al.*, 2004), camptothecin (Parsons *et al.*, 2004), UV (Birrell *et al.*, 2001), and ionizing radiation (Bennett *et al.*, 2001), which is excerpted in Table I and is shown in Fig. 6.

As an alternative to Cluster 3.0 and Java Treeview, Spotfire (<http://www.spotfire.com>) is a web-based software program useful for clustering analysis and visualization. In particular, Spotfire offers a functional genomics platform (Spotfire DecisionSite for Functional Genomics) that contains among many other options, a hierarchical clustering tool. While Spotfire does require a yearly license to be purchased in order to use the software, it is a very user-friendly system and requires virtually no understanding of statistics, bioinformatics, or programming. Users load data from an Excel file and then choose the option “hierarchical clustering” under the Data tab. The program will then offer a prompt to users, asking if they wish the data to be normalized before the clustering analysis. Users are also asked to define which columns in their data set they would like to cluster and which of five different clustering methods and six different similarity measures they would like to use on their data. Once generated, the final visualized cluster can be exported directly as a PowerPoint file.

Genome-Wide Screens for Suppressors of Genomic Instability

As indicated previously, the yeast deletion mutant collection is a useful tool for screening for events that affect growth rate. Mutations that confer a 10-fold decrease in growth are readily detectable either by pinning methods or by competitive growth assays. However, events that occur at low frequency, such as mutations, recombination, or gross chromosomal rearrangements are difficult, if not impossible to detect, by these methods. For example, a mutant displaying a 10-fold increase in mutation rate would produce a positive event only once in 10^5 to 10^6 cells. This number is simply too small to reliably detect in the number of cells that can be transferred by a high-density replica pinning device, or that can be examined in a competitive growth experiment. One means of circumventing this problem is to use strategies that allow the examination of larger numbers of cells. Rather

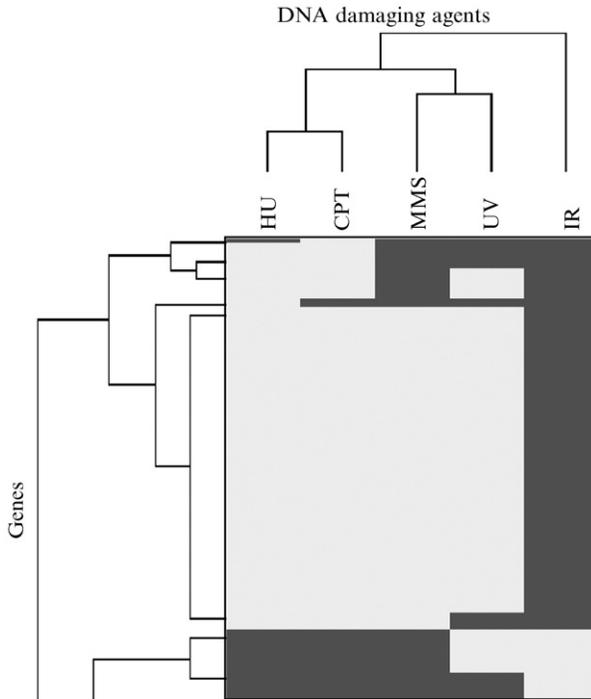


FIG. 6. An example of the output of clustering analysis performed using Cluster 3.0 and visualized using TreeView, as described in the text. Clustering was performed with data from screens with DNA damaging agents, as excerpted in Table I, and a section of the clustergram is presented. The rows are the deletion mutant array genes and the columns are the DNA damaging agents. DNA damaging agent sensitivity for a given gene deletion mutant is indicated by a dark bar at the intersection of the gene row and the DNA damaging agent column. The trees indicate the similarity of the sensitivity profiles of the DNA damaging agents and the genes. In this example, the profile of HU is more similar to that of CPT than it is to that of IR.

than screening for a particular event in the small number of cells that can be replica pinned, it is possible to screen patches of larger numbers of cells. An example of this strategy is the recent screen of the gene deletion collection for mutants that confer increased forward mutation rates (Huang *et al.*, 2003). In this screen, each deletion mutant was patched on solid media and then replica-plated to media containing canavanine. Canavanine resistant colonies result from mutations within the *CAN1* gene, and are readily scored. This strategy was particularly straightforward, as the *CAN1* gene necessary for the screen is already present in the strains that make up the gene deletion collection. However, the strategy can

readily be extended to utilize other markers of chromosomal events by using the SGA methodology to introduce the relevant marker into an array of gene deletion mutants. For example, Smith *et al.* introduced markers for a gross chromosomal rearrangement assay into the deletion collection and performed a genome-wide screen using a patching strategy (Smith *et al.*, 2004). In principle, any marker that could be used in a screen can be introduced into the gene deletion collection, including markers to assay different types of recombination, as indicated later. With the exception of minor changes in the selective media, the procedure is as presented schematically in Fig. 4.

Using SGA to Cross Reporters into Deletion Mutant Arrays

1. The strain that is typically used to make crosses with the gene deletion collection is Y5563 (*MAT α can1 Δ ::MFA1pr-HIS3 lyp1 Δ ura3 Δ 0 leu2 Δ 0 his3 Δ 1 met15 Δ 0*).

2. In most instances a PCR strategy can be used to introduce the reporter of interest. In this example, we use PCR to amplify a reporter that has been used to assay recombination between direct repeats (Smith and Rothstein, 1999). This reporter contains two mutant alleles of *leu2* flanking a *URA3* gene. Following amplification by PCR the *leu2 Δ EcoRI-URA3-leu3 Δ BstEII* reporter is introduced into Y5563 using a standard lithium acetate transformation technique (Gietz and Woods, 2002). Strains carrying the recombination reporter are selected on SD-Ura. Integration into the *leu2* locus is confirmed by PCR.

3. Using the standard SGA procedure (Tong and Boone, 2006; Tong *et al.*, 2001, 2004) the resulting strain, which carries the recombination reporter, is crossed to a *MATa* gene deletion collection array by pinning the recombination strain to YPD medium, pinning the gene deletion array on top of the recombination strain, and incubating for 1 day at 30°. Diploids are selected by pinning to SD/MSG-Ura + G418 and incubating for 2 days at 30°.

4. Diploids are sporulated by pinning to sporulation medium and incubating for 5 days at 22°.

5. Spores are germinated and haploids selected by pinning to SD-His/Arg/Lys/Ura + canavanine + thialysine plates and incubating for 2 days at 30°. The haploid selection is repeated by pinning again to SD-His/Arg/Lys/Ura + canavanine + thialysine and incubating for 1 day at 30°.

6. Haploids carrying the gene deletion and the recombination reporter are selected by pinning to SD/MSG-His/Arg/Lys/Ura + canavanine +

thialysine + G418 plates. The resulting strains are then screened for increased recombination frequency.

Screening Using a Patch Assay

1. Use a wild type strain containing the recombination reporter (or other assayable marker) as a negative control and if possible, a known mutant as a positive control.

2. Using a sterile wooden stick, streak the strains to be screened (the deletion mutant array following introduction of the reporter), and the control strains, for single colonies on SD-Ura (or other selection for the reporter to be assayed, as appropriate).

3. Pick a single colony and make an even patch, approximately 1 cm by 1 cm, on YPD. Pick another single colony of the same strain and make a duplicate patch. We find it helpful to include the positive and negative control on each plate.

4. Grow the patches for 24 h at 30°.

5. Using sterile velveteen, replica plate the duplicate patches onto SD-Leu (or the appropriate selection for your reporter system) to select for recombinants. Grow the patches for 36 h at 30°. An example of the patch assay with the recombination reporter is shown in Fig. 7.

6. Identify positives by comparing with the positive and negative controls. Both of the duplicates should score similarly.

7. The strains identified as positives should then be confirmed by a suitable assay. In the example of a recombination screen a fluctuation test would be used (Spell and Jinks-Robertson, 2004).

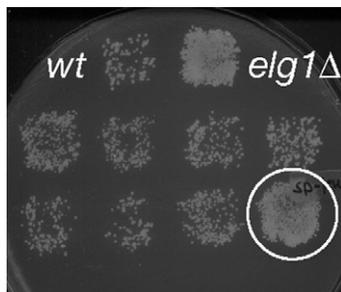


FIG. 7. Patch assay for increased recombination rate. Single colonies were streaked on YPD to form a patch. Following 24 h of growth the patches were replica-plated to the SD-Leu plate shown to detect recombinants. Wild-type and *elg1Δ* strains were included as negative and positive controls, respectively. The patch scored as positive is circled.

Media Recipes

The following recipes are for plates; for liquid media leave out the agar.

YPD: Per liter: 120 mg adenine, 10 g yeast extract, 20 g peptone, 20 g bacto agar (BD Difco), 950 ml water. After autoclaving, add 50 ml of 40% glucose solution.

Drop-out mix (DO): 3 g adenine, 2 g uracil, 2 g inositol, 0.2 g para-aminobenzoic acid, 2 g alanine, 2 g arginine, 2 g asparagine, 2 g aspartic acid, 2 g cysteine, 2 g glutamic acid, 2 g glutamine, 2 g glycine, 2 g histidine, 2 g isoleucine, 10 g leucine, 2 g lysine, 2 g methionine, 2 g phenylalanine, 2 g proline, 2 g serine, 2 g threonine, 2 g tryptophan, 2 g tyrosine, 2 g valine *minus* the indicated supplements.

SD-Ura: Per liter: 6.7 g yeast nitrogen base w/o amino acids (BD Difco), 2 g amino-acids supplement powder mixture (DO – Ura), 100 ml water. Add 20 g bacto agar to 850 ml water. Autoclave separately. Combine and add 50 ml 40% glucose.

SD-Leu: Per liter: 6.7 g yeast nitrogen base w/o amino acids (BD Difco), 2 g amino-acids supplement powder mixture (DO – Leu), 100 ml water. Add 20 g bacto agar to 850 ml water. Autoclave separately. Combine and add 50 ml 40% glucose.

SD/MSG-Ura+G418: Per liter: 1.7 g yeast nitrogen base w/o amino acids or ammonium sulfate (BD Difco), 1 g MSG (L-glutamic acid sodium salt hydrate), 2 g amino-acids supplement powder mixture (DO – Ura), 100 ml water. Add 20 g bacto agar to 850 ml water. Autoclave separately. Combine autoclaved solutions, add 50 ml 40% glucose, cool to ~65°, add 1 ml G418 (Geneticin, Invitrogen; 200 mg/ml in water) stock solution.

Sporulation Medium: Per liter: 10 g potassium acetate, 1 g yeast extract, 0.5 g glucose, 0.1 g amino-acids supplement powder mixture for sporulation (contains 2 g histidine, 10 g leucine, 2 g lysine, 2 g uracil), 20 g bacto agar to 1 liter water. After autoclaving, cool medium to ~65°, add 250 μ l of G418 stock solution.

SD-His/Arg/Lys/Ura + canavanine + thialysine: Per liter: 6.7 g yeast nitrogen base w/o amino acids (BD Difco), 2 g amino-acids supplement powder mixture (DO – His/Arg/Lys/Ura), 100 ml water. Add 20 g bacto agar to 850 ml water. Autoclave separately. Combine autoclaved solutions, add 50 ml 40% glucose, cool medium to ~65°, add 0.5 ml canavanine (L-canavanine sulfate salt, Sigma, C-9758; 100 mg/ml in water) and 0.5 ml thialysine [S-(2-aminoethyl)-L-cysteine hydrochloride, Sigma, A-2636; 100 mg/ml in water] stock solutions.

SD/MSG-His/Arg/Lys/Ura + canavanine + thialysine + G418: Per liter: 1.7 g yeast nitrogen base w/o amino acids or ammonium sulfate, 1 g MSG, 2 g amino-acids supplement powder mixture (DO – His/Arg/Lys/

Ura), 100 ml water. Add 20 g bacto agar to 850 ml water. Autoclave separately. Combine autoclaved solutions, add 50 ml 40% glucose, cool medium to $\sim 65^\circ$, add 0.5 ml canavanine, 0.5 ml thialysine, and 1 ml G418.

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