Knocking out multigene redundancies via cycles of sexual assortment and fluorescence selection

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Phenotypes that might otherwise reveal a gene's function can be obscured by genes with overlapping function. This phenomenon is best known within gene families, in which an important shared function may only be revealed by mutating all family members. Here we describe the 'green monster' technology that enables precise deletion of many genes. In this method, a population of deletion strains with each deletion marked by an inducible green fluorescent protein reporter gene, is subjected to repeated rounds of mating, meiosis and flow-cytometric enrichment. This results in the aggregation of multiple deletion loci in single cells. The green monster strategy is potentially applicable to assembling other engineered alterations in any species with sex or alternative means of allelic assortment. To test the technology, we generated a single broadly drug-sensitive strain of *Saccharomyces cerevisiae* bearing precise deletions of all 16 ATP-binding cassette transporters within clades associated with multidrug resistance.

Robustness of biological systems to genetic insults is widespread. Synergistic sick or lethal interactions are observed for over 75% of nonessential genes in *S. cerevisiae*[1,2]. Moreover, interactions involving three or more genes (for example, the seven-gene genetic interaction involving oxysterol-binding protein genes[3]) are likely to outnumber pairwise genetic interactions[1]. Gene families with members related by sequence may frequently harbor a multigene interaction, but few of the 80 yeast gene families with six or more genes[4] (**Supplementary Table 1**) have been entirely deleted to identify important underlying shared functions.

Functional redundancy complicates development of pharmaceuticals. For example, ATP-binding cassette (ABC) transporters overlap in the drugs they export. To isolate the activities of individual transporters, a yeast strain (AD12345678; here termed AD) was previously generated with mutations in nine genes, including seven ABC transporter genes[5]. This strain is hypersensitive to many compounds, facilitating many studies of drug mechanism[6] and offers a simplified genetic background for characterizing exogenous transporters[7]. However, the two ABC transporter gene clades implicated in drug efflux contain 16 genes[8,9], motivating additional deletions in this family.

In yeast, genes are deleted by replacement with a selectable marker via homologous recombination[10]. However, engineering strains bearing many unlinked deletions presents challenges. (i) If deletions are introduced sequentially, the time required scales linearly with the number of target genes. (ii) If specific subsets of deletions are lethal in combination, different 'dead ends' may be reached depending on the order of deletion, so that achieving the greatest tolerable number of deletions may be impracticable. (iii) There are a limited number of useful selectable markers.

All commonly used methods suffer from the first two problems, but in some methods the third problem has been addressed by removing markers before reuse. Unfortunately, each marker-removal method has additional problems. In the *hisG* method, flanking bacterial *hisG* sequences recombine to excise the deletion cassette[11], but remnant *hisG* sequences accumulating in the genome cause mistargeting of subsequently introduced knockout fragments[12,13]. The Cre-*lox* method improves marker excision efficiency and leaves a shorter remnant sequence[14,15]. However, Cre recombinase has been observed to catalyze massive genome rearrangements between nonadjacent *loxP* sites[15], for example, in glucose transporter deletion strains[16] (E. Boles, personal communication). In the *delitto perfetto* method, a short fragment with homology specific to each locus is used for excision of each marker[17]. Because yeast transformation is required twice

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RECEIVED 18 OCTOBER; ACCEPTED 8 DECEMBER 2010; PUBLISHED ONLINE 9 JANUARY 2011; DOI:10.1038/NMETH.1550

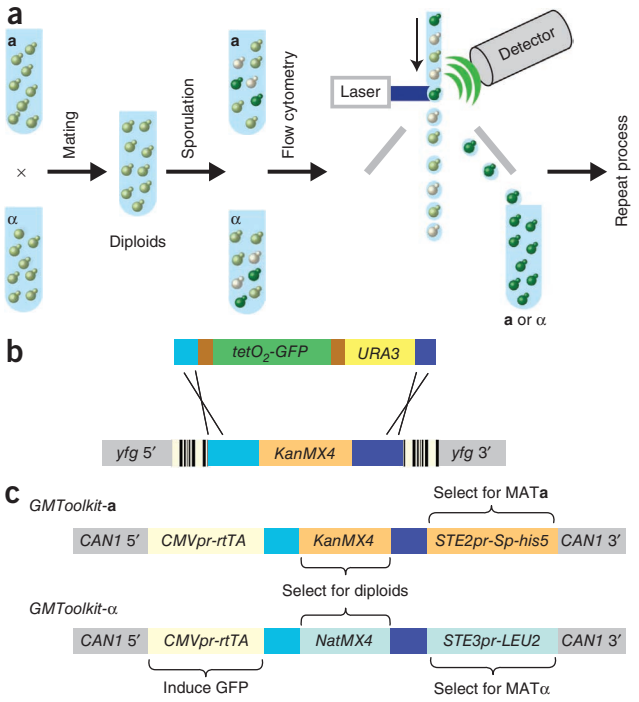

Figure 1 | Design of the green monster process. (**a**) Schematic overview of the process. In yeast, crossing different haploid single mutants generates no-deletion (off-white), one-deletion (light green) and two-deletion (dark green) cells. From this mixture, flow cytometry is used to enrich for two-deletion cells. Higher-order multimutants are assembled via repeated rounds of sexual assortment and enrichment. (**b**) In this process a universal *GFP* deletion cassette replaces *KanMX4* in a target gene (*yfg*) via recombination within *Kan* subsequences internal to the flanking barcodes. The inducible $tetO_2$ promoter allows titration of GFP expression. Transcriptional terminators (brown) and the *Kan* promoter (light blue) and terminator (dark blue), each derived from the *Ashbya gossypii TEF* gene, are shown. The transformation marker is *URA3*. (**c**) *GMToolkits*, inserted at the *CAN1* locus, contain *rtTA*[21] and either *KanMX4* and *STE2pr-Sp-his5* (*GMToolkit*-**a**) or *NatMX4*[20] and *STE3pr-LEU2* (ref. 19) (*GMToolkit*-α).

per cycle, this method is time-consuming. Here we describe the 'green monster' technology which addresses all three problems. Using this technology, we generated broadly drug-sensitive 'ABC16-monster' strains lacking 16 ABC transporter genes in clades implicated in multidrug resistance.

RESULTS

The green monster strategy

The core idea of the green monster strategy is that multiple genetic alterations, each constructed in a separate strain and each labeled with a quantitatively selectable marker such as *GFP*, can be assembled into a single strain. Assembly is done via repeated rounds of sexual assortment and enrichment of progeny bearing an ever-increasing number of altered loci (**Fig. 1a**). The strategy is general to any species for which (i) individual loci can be engineered and marked to allow selection or efficient screening on the basis of gene dosage and (ii) alleles can be reassorted via mating or its equivalent (such as viral transfection or bacterial conjugation). Here we implement the strategy for *S. cerevisiae*.

The initial ingredient is a set of 'ProMonster' strains, each carrying an identical inducible *GFP* reporter replacing one of the target genes. Our universal deletion cassette employs the S65T variant of GFP (**Fig. 1b**). To facilitate repeated sexual assortment of these deletions, ProMonsters of mating types (MATs) **a** and α carry the *GMToolkit*-**a** and *GMToolkit*-α constructs, respectively, at the *CAN1* locus (**Fig. 1c**; GM stands for green monster). Each *GMToolkit* contains: (i) one of two markers allowing selection of haploids of the appropriate mating type, either *Schizosaccharomyces pombe his5* (known to complement *S. cerevisiae his3* mutations)[18] under the control of the MAT**a**-specific *STE2* promoter or *LEU2* driven by the MATα-specific *STE3* promoter[19]; (ii) one of two drug-selectable markers, either *KanMX4* conferring resistance to G418 or *NatMX4* conferring resistance to nourseothricin[20], collectively allowing selection for MAT**a**/α diploid cells carrying both *GMToolkit* constructs; and (iii) the Tet activator *rtTA*[21] for fluorescent protein induction to enable flow-cytometric enrichment. Deletion strains can either be pooled (in the *en masse* process) or be isolated and genotyped before each cross to maintain specific deletions with reduced fitness in the population.

Simulation of the green monster process

Fluorescence intensity varies even in isogenic cell populations. To gauge the impact of this variation on our ability to separate cells by *GFP* gene dosage, we performed Monte Carlo simulations of the *en masse* process. Simulations initiated with 24 theoretical ProMonster strains of each mating type corresponding to 24 mutually unlinked genes yielded a population with 99% of cells missing all 24 genes in 12 rounds (**Fig. 2a**). It may seem counter-intuitive that cells with 24 copies of *GFP* could be separated from cells carrying 23 copies of *GFP* when GFP expression (and therefore GFP intensity) arising from each locus can vary among cells by 50%; however, the extreme high-intensity tail of the GFP intensity distribution should be dominated by the cells carrying the most *GFP* copies.

To combine deletions on the same chromosome, a meiotic cross-over must occur between deleted loci. When simulating an extreme case in which all deletions were on one chromosome spaced ~15 kilobases (kb) apart, the final multimutant dominated the population in 16 rounds (**Fig. 2b**) as opposed to 12 rounds if each deletion locus was assorting independently. Therefore, genetic linkage between deletion loci is not problematic (and may be an advantage in later rounds in that once linked, altered loci will tend to stay together).

Generating and testing green monster reagents

To implement the green monster strategy, we constructed universal GFP deletion cassettes (**Fig. 1b**; $tetO_2pr$-*GFP*, where $tetO_2pr$ refers to a promoter bearing two copies of the *tetO* regulatory unit) as well as *GMToolkit*-**a** and *GMToolkit*-α (**Fig. 1c**). Given the existing library of *KanMX4* deletion strains, our universal deletion cassette enables targeting of any locus without any locus-specific reagents (by virtue of flanking sequences with homology to *KanMX4*). This replacement retains the barcode sequences that uniquely identify each locus in this library. In testing this strategy (Online Methods), we correctly inserted the *GFP* cassette in all Ura^+ G418-sensitive transformants (24 transformants; Ura^+ indicates the ability to grow without supplementary uracil). We also generated a cassette with *HphMX4* (ref. 20) instead of *URA3*. Should GFP expression saturate the production

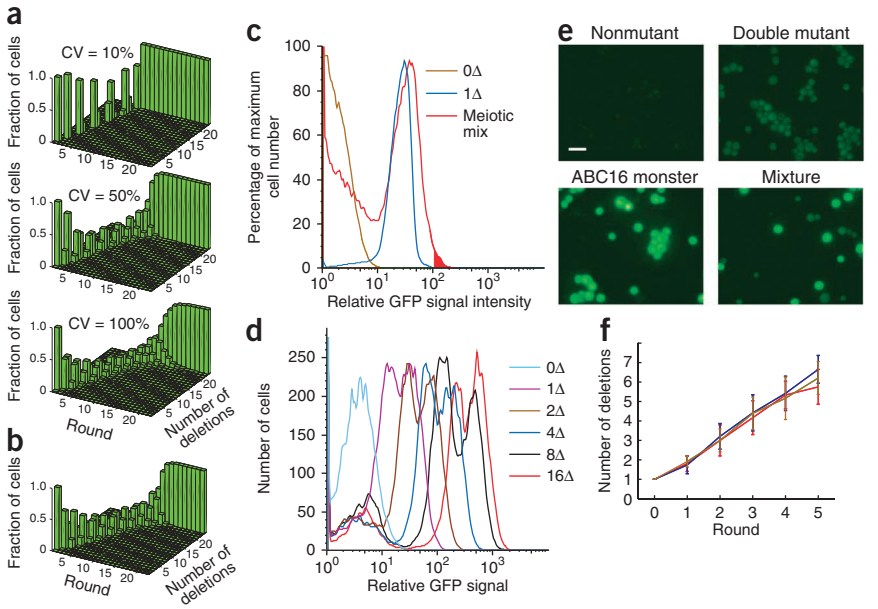

Figure 2 | Demonstration of the green monster process. (**a**) Simulations showing that >99% of a cell population accumulate all 24 deletions in eight (top), 12 (middle) or 19 rounds (bottom), with greater efficiency for lower coefficient of variation (CV) of GFP signal intensity (achievable using internal standard to control for noise). (**b**) Simulation showing that 24 linked deletions with the meiotic cross-over probability between adjacent loci of 5% can be assembled in 16 rounds when the GFP signal intensity CV is 50%. (**c**) Histograms illustrating the results of cell sorting for no-*GFP* cells (0Δ), single-*GFP* cells (1Δ) and a haploid 'meiotic mix' resulting from a cross of two single-*GFP* strains, with an expected 1:2:1 ratio of no-*GFP*, one-*GFP* and two-*GFP* (2Δ) cells. The brightest 1% of the cells in the meiotic mix were collected (red filled area). (**d**) GFP fluorescence intensity (arbitrary units) of multimutants. Histograms are shown for no-*GFP*, 1-*GFP*, 2-*GFP*, 4-*GFP*, 8-*GFP* and 16-*GFP* 'ABC16-monster' cells (isogenic populations). (**e**) Fluorescence micrographs showing nonmutant cells, double-mutant cells, ABC16-monster cells, and a mixture of double-mutant and ABC16-monster cells. Identical exposure, brightness, and contrast settings were used for images. Scale bar, 10 μm. (**f**). Average deletion numbers for the *en masse* green monster process from three independent processes are plotted. Error bars, s.d. From Round 1 to Round 5, n = 21, 23, 24, 24, 24 (red); n = 23, 24, 24, 24, 23 (blue); n = 24, 24, 23, 24, 34 (brown).

capacity of cells or become toxic, the $tetO_2$ promoter allows GFP expression to be reduced by lowering doxycycline concentration. For essential target genes, $tetO_2$*pr-GFP* cassettes can be linked to temperature-sensitive alleles[22] or other informative alleles[23].

To assess each component, we mated a MAT**a** strain carrying the *HphMX4* $tetO_2$*pr-GFP* cassette with MATα cells carrying both a *URA3* $tetO_2$*pr-GFP* cassette at a different unlinked locus and *GMToolkit*-α (Online Methods). Then we sporulated the resulting diploids. Assuming normal meiotic segregation of two unlinked genes, we expected MATα GMToolkit-α cells selected from this meiotic mix to be a 1:2:1 mixture of no-*GFP*, one-*GFP* and two-*GFP* cells. We collected the most fluorescent cells (**Fig. 2c**) and genotyped them using *HphMX4* and *URA3* markers. Of 60 colonies derived from unsorted cells, 25% were double mutants as expected, whereas 92% of 60 colonies derived from sorted cells were double mutants, demonstrating selection of multimutants using GFP fluorescence intensity.

Deleting 16 ABC transporter genes

To test the scalability of the green monster process, we targeted all 16 genes in the ABCC and ABCG clades in the *S. cerevisiae* ABC transporter family (these clades contain multiple genes associated with multidrug resistance)[8,9]. For each of the 16 targets, we generated both MAT**a** and MATα ProMonster strains. When we combined all strains in a pool and subjected them to one round of the process (**Supplementary Fig. 1**), 12 of the resultant haploids (100%) were double mutants (~25% would be expected from random segregation). We crossed genotyped double-mutant strains, either in pools or individually, with opposite mating-type strains (**Supplementary Figs. 1** and **2**). In 16 out of 21 crosses throughout the lineage, the average number of deletions in sorted strains exceeded the number expected for unsorted strains (**Supplementary Table 2**). This measure of enrichment is conservative in that expectations did not account for potential underrepresentation of higher-order mutants owing to reduced fitness. Because many crosses can proceed in parallel, the total time required scaled not with the total number of crosses but with the greatest number of rounds in any lineage from ProMonster to the final multimutant.

We obtained 'ABC16 monsters' with all 16 transporter genes deleted in no more than 11 rounds along any lineage. Because each round of the green monster process requires 13 d (Online Methods), 143 d would thus be sufficient for construction of the ABC16 monsters following this protocol. We estimate that the green monster process reduced the time required for strain construction using the *hisG*, Cre-*lox* or *delitto perfetto* method by about 40%, without taking into account other limitations of these approaches (Online Methods). We confirmed both the absence of coding sequences and the presence of the deletion cassette using PCR at all 16 loci. Despite the above-mentioned ectopic reciprocal recombination seen with the Cre-*lox* method, the ABC16-monster had no recognizable karyotype defects (**Supplementary Fig. 3**). Consistent with the absence of ectopic rearrangement, PCR primers external to the deletion cassette loci for each of the targeted loci amplified fragments of the expected size (**Supplementary Fig. 4** and Online Methods). Analysis of the ABC16 monster and other strains showed a near-linear correlation between fluorescence intensity and number of deletions, both in flow cytometric analysis and in micrographs (**Fig. 2d**,**e**). GFP expression in these cells, even with high levels of the inducer doxycycline, did not affect growth rates (**Supplementary Table 3**).

To confirm the effectiveness of flow cytometry in enriching for strains with more than a few deletions, we crossed the ABC16 monster with a nonmutant strain and genotyped both sorted and unsorted haploid progeny resulting from one round of the green monster process. By sorting the brightest 1% of cells, we obtained a greater average of 9.6 ± 0.3 deletions (s.e.m.; n = 24 for all samples) as compared with 7.6 ± 0.5 deletions for the unsorted

sample ($P = 0.002$). Sorting the brightest 0.1% of cells achieved an average of 10.3 ± 0.4 deletions ($P = 0.0002$).

To evaluate the generality of the green monster process, we applied it to assemble another 'green monster' comprising deletions in six genes that are unrelated to ABC transporters: *YER042W*, *YCL033C*, *YDL242W*, *YDL227C* (*HO*), *YKL069W* and *YOL118C* (V.M.L. and V.N.G., unpublished data). We generated this six-deletion strain in three rounds (**Supplementary Table 4**). Because *YDL242W* and *YDL227C* are separated by only 27 kb, we used these loci to assess whether recombination is elevated between nearby deletion cassettes. PCR using primer pairs external to each *GFP* cassette yielded bands of the expected sizes, and PCR designed to detect cassette-mediated recombination between loci did not detect any rearrangement (**Supplementary Fig. 5** and Online Methods).

Evaluating the *en masse* green monster process

The processes described above exploited both GFP fluorescence and genotyping to enrich for multimutants. To evaluate the green monster process as implemented without the benefit of enrichment via genotyping, we carried out three independent lines of the process (**Supplementary Fig. 6**) such that we took heterogeneous pools of deletion strains emerging from each fluorescence-based selection directly into the next round and mated them together *en masse*. The green monster process was highly efficient in selecting the correct cell type (haploid or diploid) in each step (**Supplementary Table 5**). To monitor progress in terms of number of deletion alleles, we genotyped a sample of strains from the sorted pools. The average deletion numbers ascended in each round to reach six in only five rounds of the green monster process (**Fig. 2f** and **Supplementary Table 6**). Moreover, the final populations included eight- and nine-deletion strains. The rapid *en masse* option for allele assembly without isolating and genotyping strains can reduce each round time from 13 d to 11 d and reduce the number of processes that are run in parallel. It could be widely applicable, particularly where multimutant fitness effects are limited. Single-mutant fitness defects are limited for the majority of deletions[24] and can be further limited via the use of reduced-function[23] or conditional[22] alleles, or by ectopic expression of target genes during strain construction. However, PCR-based genotyping can inform the choice of crosses to maintain the presence of alleles with reduced fitness or fluorescence intensity. Such genotyping is not labor-intensive and also reveals progress during the process.

Testing drug sensitivity for ABC16 monsters

To compare drug sensitivity of the ABC16 monster with the previous drug-hypersensitive AD strain[5], we measured drug sensitivity in response to ~400 drugs previously used in human clinical trials (US National Institutes of Health Clinical Collection; **Supplementary Table 7**). The ABC16 monster exhibited sensitivity to a comparable number of drugs as the AD strain (**Fig. 3a** and **Supplementary Table 8**). The strains were complementary in that, for 34% of all drugs tested, only one of the two hypersensitive strains exhibited sensitivity. The ABC16 monster should, for example, be particularly useful for studying the mechanisms of action for clotrimazole, benidipine, cisapride and perospirone, each of which inhibited the growth of the ABC16 monster by more than 75% but had little effect on the growth of AD (**Supplementary Table 8**). Resistance to some drugs, observed for both AD and ABC16-monster strains (for example, carmofur and rimcazole), may arise from deletion of one transporter gene that triggers resistance via compensatory upregulation of other transporters[25].

To evaluate the effects of eliminating multiple genes with overlapping function ('multigene redundancy'), we compared concentrations at which growth was inhibited by 50% (IC_{50} values) of the ABC16 monster with those of single mutants for compounds previously used[7] to characterize ABC transporters (**Fig. 3b**,**c**, **Supplementary Table 9** and Online Methods). At least three patterns of drug response emerged.

In the first pattern, exemplified by tamoxifen and carbonyl-cyanide *m*-chlorophenylhydrazone (CCCP), the ABC16 monster was more sensitive than nonmutant and most single-mutant strains tested but on par with the most sensitive single mutant. This suggests the existence of one or two major transporters for each drug (for example, Pdr5p and Pdr11p for tamoxifen and Snq2p for CCCP; **Fig. 3b**), with little compensatory activity provided by the other transporters.

The second drug response pattern was exemplified by fluconazole, ketoconazole, miconazole and itraconazole. The ABC16 monster was highly sensitive, similarly to the AD strain[7], but

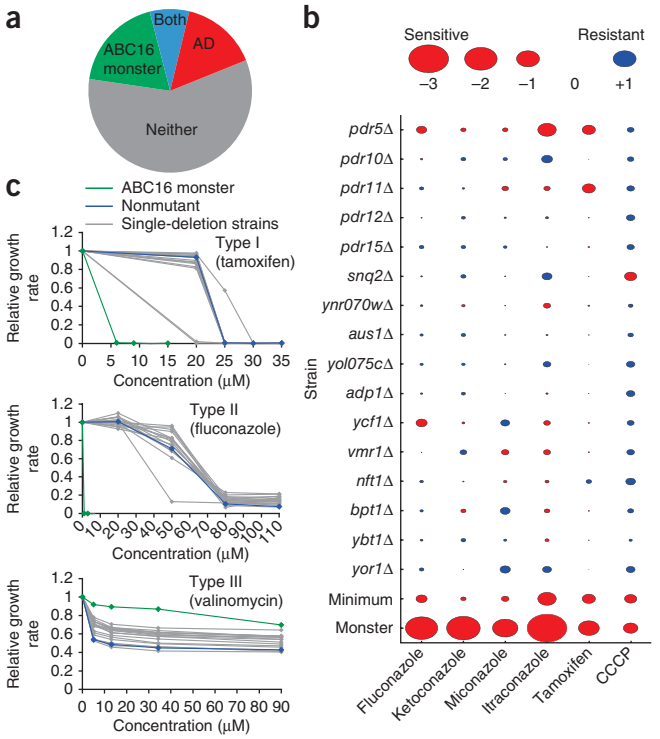

Figure 3 | Hypersensitivity of the ABC16 monster to drugs. (**a**) Number of drugs to which the ABC16 monster or the previously described drug-hypersensitive AD strain is sensitive compared to wild type. (**b**) IC_{50} values for single-mutant drug sensitivity and for ABC16-monster drug sensitivity relative to that of the corresponding nonmutant-drug combination as indicated in the legend (top). The minimum value among the relative IC_{50} values for single mutants is indicated for comparison with the relative IC_{50} of the ABC16 monster. (**c**) Exponential growth rates of the ABC16-monster (green), nonmutant (blue) and single-deletion strains (gray) as a function of tamoxifen, fluconazole and valinomycin concentration.

single mutants other than *pdr5*Δ did not have marked sensitivity (**Fig. 3b**,**c**). This suggests that transporters other than Pdr5p are involved in transporting these drugs, such that the effect of deleting each transporter alone is diminished by overlapping activity among the others. The fitness of the ABC16 monster was lower than expected from a multiplicative model of independent effects[26] across a range of concentrations for each of these azole drugs, indicating synergistic genetic interaction (**Supplementary Table 10**).

The third pattern of drug response was exemplified by valinomycin, an ionophore that disrupts mitochondrial function. The ABC16 monster was unexpectedly more resistant than nonmutant or any single mutant to valinomycin (**Fig. 3c**). As 13 single mutants had slight resistance compared to nonmutant cells, the monster may simply have the aggregated effects of single deletions. Previously, increased resistance in ABC-transporter deletion strains has been observed for substrates of Pdr5p, Snq2p and Yor1p[25]. In this previous example, specific efflux pumps responsible for the resistance had been known already. However, our deletion of 16 single mutants potentially defective in drug transport did not reveal an obvious candidate transporter for valinomycin. Potential mechanisms include compensatory upregulation of other proteins capable of valinomycin transport, (for example, antiporters of the major facilitator superfamily) and import of valinomycin by two or more ABC transporters, as suggested for other substrate-transporter combinations[27].

Taken together, our analysis of drug sensitivity in ABC16-monsters showed that the removal of confounding activities in large gene families with overlapping function can reveal phenotypes that would be otherwise obscured.

DISCUSSION

The green monster process was enabled by quantitative markers such as *GFP* that allow simultaneous selection for the presence of multiple altered loci. Markers that permit selection for gene dosage have been used to select for segmental amplification at a given locus but not to select for alleles at multiple distinct loci. The marker system used in the green monster process to 'drive' cells through the sexual cycle may enable other studies, such as laboratory evolution using a sexually recombining cell population.

An ABC16-monster strain we constructed was complementary to a previously constructed drug-hypersensitive strain (AD) in that it revealed sensitivity that is not present in either wild-type or the AD strain for 19% of all drugs tested. ABC16-monster strains should facilitate characterization of diverse compounds that are efficiently exported from wild-type or AD yeast cells. Somatic or germline mutations in at least ten human ABC transporter genes affect drug sensitivity[28]. Yeast has proven to be useful for characterizing polymorphisms in the human *ABCB1* gene[29]. Given that most known human drug transporters belong to the ABCC or ABCG subfamilies that we entirely deleted in the ABC16 monster, this strain provides a cleaner genetic background for identifying human ABC transporter substrates or inhibitors.

The green monster process has advantages compared to some other technologies for strain construction: it can be used to engineer multiple loci in each round; it can avoid local maxima in the number of tolerable deletions by sampling many construction paths in parallel; and it circumvents constraints imposed by the limited number of useful selectable markers. The process of assembling green monsters yields many more intermediate strains than would be produced in more linear construction approaches. These intermediate strains could prove useful for exploring complex genotype-phenotype relationships.

The green monster process has the same limitations as all other strategies. For essential genes (either individually or in combination) the process cannot proceed without the use of hypomorphic, conditional or ectopically expressed 'covering' alleles. As other methods, it is subject to the appearance of adaptive mutations. However, the green monster process may be subject to aneuploidies (present in at least 8% of deletion strains[30]) to a lesser extent, given that the strains produced using this process must repeatedly survive meiosis.

The green monster process has limits that other methods do not. Combining otherwise-viable deletions in genes required for mating, sporulation or *GFP* expression will be problematic. The total number of such genes is unknown, but we expect it is fewer (perhaps much fewer) than 15% of all genes. We saw no evidence for ectopic recombination after isolation of the original colony, outgrowth to generate a frozen glycerol stock, and subsequent thawing and outgrowth for analysis of genomic integrity. However, green monster strains are in theory more susceptible to ectopic recombination, so strains should be examined periodically for instability.

The core requirements of the green monster technology, a quantitatively selectable marker and the ability to combine alleles through sexual assortment or its equivalent, are met by many species. Although the specific schemes will necessarily differ, we envision application of the strategy in three model organisms (an overview in *Escherichia coli*, *Caenorhabditis elegans* and *Mus musculus* is available in **Supplementary Fig. 7**).

Finally, the green monster strategy could be readily extended to allow insertion of exogenous genes and therefore permit the construction of exogenous or synthetic pathways in yeast or other model organisms.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/naturemethods/.

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

ACKNOWLEDGMENTS

This work was supported by US National Institutes of Health grants R01 HG003224 and R21 CA130266 to F.P.R. O.D.K. was supported by a National Research Service Award fellowship from the National Institutes of Health–National Human Genome Research Institute. Micrographs were generated at Nikon Imaging Center at Harvard Medical School. We are grateful for yeast strains from C. Boone (University of Toronto) and A. Goffeau (Université catholique de Louvain), and for advice and assistance from M. Al-Shawi, B.J. Andrews, C. Baisden, R. Balasubramanian, W. Bender, C. Boone, R. Brost, S. Buratowski, D. Chowdhury, G.M. Church, D. Coen, E. Craig, E. Elion, M. Fenerjian, D. Gibson, J. Glass, A. Goffeau, R. Grene, C. Hutchison, S. Iwase, M. Johnston, B. Karas, K. Kono, K. Kuchler, J. Li, D. Morgan, S. Moye-Rowley, J. Murai, S. Oliver, F. Ozbek, Y. Pan, D. Pellman, A. Ramon, J. Rine, A. Rowat, P. Silver, H. Smith, M. Springer, K. Struhl, C. Tagwerker, M. Takahashi, B. Turcotte, D. Weitz and S. Yoshida; members of the Roth Lab, especially M. Tasan, J. Mellor, J. Komisarof, J. MacKay, A. Derti and S. Komili; and members of the Dana-Farber Center for Cancer Systems Biology, especially P. Braun, A. Dricot, D. Hill, Q. Li and H. Yu.

AUTHOR CONTRIBUTIONS

Y.S. and F.P.R. developed the green monster method and prepared the manuscript; R.P.S.O., A.H., J.L. and Y.S. measured drug sensitivity; R.M. analyzed growth curves; O.D.K. simulated the process; L.V.Z., C.N. and G.G. advised on method design; A.H.Y.T., V.M.L., V.N.G. and M.V. provided reagents and advice; W.C. and L.P. provided technical support; P.S. and Y.S. performed flow cytometry.

COMPETING FINANCIAL INTERESTS
The authors declare no competing financial interests.

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ONLINE METHODS

Yeast strains. All yeast strains have the background genotype of BY4741 (*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*) or BY4742 (*MAT*α *his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*)[31] except AD12345678 (*MAT*α *PDR1–3 ura3 his1 yor1Δ::hisG snq2Δ::hisG pdr5Δ::hisG pdr10Δ::hisG pdr11Δ::hisG ycf1Δ::hisG pdr3Δ::hisG pdr15Δ::hisG*)[5]. The GMToolkit-**a** (RY0146) and GMToolkit-α (RY0148) strains have the background *MAT*α *lyp1Δ his3Δ1 leu2Δ0 ura3Δ0 met15Δ0* and have *can1Δ::GMToolkit-***a** (*CMVpr-rtTA KanMX4 STE2pr-Sp-his5*) and *can1Δ::GMToolkit-*α (*CMVpr-rtTA NatMX4 STE3pr-LEU2*), respectively. *Pr* denotes promoter. *KanMX4* and *NatMX4*[20] confer resistance to 200 μg ml^{-1} G418 (Sigma-Aldrich) and 100 μg ml^{-1} nourseothricin (Nat; Werner BioAgents), respectively. The genotypes of ABC16 monsters (RY0512, RY0513, RY0521 and others) are *MAT***a** (or *MAT*α) *adp1Δ snq2Δ ycf1Δ pdr15Δ yor1Δ vmr1Δ pdr11Δ nft1Δ bpt1Δ ybt1Δ ynr070wΔ yol075cΔ aus1Δ pdr5Δ pdr10Δ pdr12Δ can1Δ::GMToolkit-***a** (or -α) *his3Δ1 leu2Δ0 ura3Δ0 met15Δ0*. Each ABC-transporter deletion contains *ADHterm-tetO$_2$pr-GFP*(S65T)*-CYC1term URA3*. *Term* denotes terminator. Control strains for drug assay (RY0566 and RY0568) were MAT**a** and MATα ProMonster strains containing the *GFP* deletion cassette in the neutral *ho* deletion locus. Except for sporulation, we grew yeast cells on rich YPD medium or on synthetic complete (SC) medium lacking the relevant auxotrophic components. We plan to share materials in adherence to the US National Institutes of Health (NIH) grant Policy on Sharing of Unique Research Resources.

Simulation of the green monster process. We implemented Monte Carlo simulation of sexual cycling and fluorescence-mediated sorting in Java (Oracle), with results visualized using Matlab (Mathworks) (codes are freely available upon request). The initial simulated MAT**a** and MATα pools each contained 24 deletion strains. At each cell sorting step, we computed GFP fluorescence intensity for each cell by summing independent samples from a Gaussian distribution for each *GFP* locus, modeling the coefficient of variation between cells of GFP fluorescence intensity from a given *GFP* locus to be 10%, 50% or 100%. We modeled cell sorting as keeping cells in the 99th percentile of GFP fluorescence intensity. We modeled deletion loci as linked or independently assorting (that is, probability of meiotic recombination between each pair of deletion loci was set to 50%). The simulation tracked a population of 100,000 cells at each step. Repeated runs gave equivalent results.

Generation of the universal *GFP* replacement cassettes. We cloned sequence encoding full-length, wild-type GFP, amplified with primers containing BamHI and ApaI sites into the BamHI and ApaI sites of pCM251 (ref. 32) to make pYOGM002a, cloned a 1.5-kb region containing *ADHterm-tetO$_2$-GFP-CYC1term* amplified from pYOGM002a into the XhoI and HindIII sites of pBluescript SK+ (Stratagene) to make pYOGM005, and cloned a 1-kb EcoRI-XbaI *URA3* PCR fragment into pYOGM005 to make pYOGM007a. We introduced a change in the *GFP* sequence in pYOGM007a to encode GFP S65T from plasmid pYOGM012. PCR with primers 5′-GGATCCCCGGGTTAATTAAGGCGC GCCAGATCTGTTTAGCTTGCCCAAGCTCCTCGAGTAAT TCG-3′ (primer A) and 5′-GGCGTTAGTATCGAATCGACA GCAGTATAGCGACCAGCATTCACGTACCGGGTAATAAC TGATATAAT-3′ produced a *KanMX4* replacement fragment. Similarly, we made the *HphMX4* (ref. 20) version of the targeting plasmid (pYOGM013). The difference was that, instead of the *URA3* fragment, we cloned a 1.5-kb *HphMX4* PCR fragment from pAG32 (ref. 20) with XbaI and NotI sites at the ends into pYOGM005. PCR with primer A and a primer with the sequence 5′-CAGTATAGCGACCAGCATTCAC-3′ produced a *KanMX4*-targeting fragment.

Construction of the *GMToolkit* constructs. For assembling *GMToolkit-***a**, we cloned a *KanMX4* PCR fragment flanked by XbaI and XhoI sites into XbaI and SalI sites of pUC18 to make pYOGM014a, and cloned a *CMVpr-rtTA*[33,34] PCR fragment flanked by KpnI and NheI sites into the KpnI and XbaI sites of pYOGM014a to make pYOGM019. From this plasmid, we amplified a fragment carrying *CMVpr-rtTA* and *KanMX4* and integrated it into the *can1Δ* locus upstream of *STE2pr-Sp-his5* in the strain Y7092 (gift from C. Boone)[19]. This integration replaced ~200 bp of the *CAN1* promoter region. For *GMToolkit-*α, we cloned a *STE3pr::LEU2* (ref. 19) PCR fragment flanked by BamHI and ApaI sites into BamHI and ApaI sites of pBluescript SK+ to make pYOGM015a, cloned a *NatMX4* (ref. 20) PCR fragment flanked by XbaI and XhoI sites into XbaI and SalI sites of pYOGM015a to make pYOGM018a, and cloned a *CMVpr-rtTA* PCR fragment flanked by NheI sites into the XbaI site of pYOGM018a to make pYOGM020. From this plasmid, we generated a targeting fragment containing *CMVpr-rtTA*, *NatMX4* and *STE3pr::LEU2* and used it to replace *STE2pr-Sp-his5* and ~200 bp of *CAN1* promoter at the *CAN1Δ* locus in strain Y7092.

Confirmation of green monster components. We targeted the *URA3-* and *HphMX4*-marked universal *GFP* replacement cassettes to several arbitrarily chosen genes *YMR139W, YGL180W, YJL164C, YNL307C* and *YJL187* as well as the 16 ABC transporter genes, using previously established MAT**a** *KanMX4* deletion strains (BY4741 background genotype)[24]. The *URA3* marker allows strains with this background to propagate without supplemented uracil. *HphMX4* confers resistance to hygromycin B (300 μg ml^{-1}; Calbiochem)[20]. Some integrants were crossed with a MATα GMToolkit strain, using the method described for generation of ProMonsters. For confirming functions of molecular tools, we mated MAT**a** cells (*yjl187cΔ::HphMX4 tetO$_2$pr-GFP*) with MATα cells (*snq2Δ::URA3 tetO$_2$pr-GFP can1Δ::GMToolkit-*α). From this cross, we selected diploids using *HphMX4* (ref. 20) and *NatMX4* (in *GMToolkit-*α). After sporulation of diploids (see 'sexual cycling of green monsters'), we selected MATα cells using SC medium without leucine (SC –Leu medium) by virtue of the *GMToolkit-*α marker *STE3pr-LEU2*, which is activated only in MATα cells. GFP expression was induced as described below in 'sexual cycling of green monsters'. Given normal meiotic segregation of two unlinked genes, resulting cells should be a 1:2:1 mixture of no-*GFP*, one-*GFP* and two-*GFP* cells, respectively. When analyzed using flow cytometry (**Fig. 2c**), the mixture of cells had a wider distribution of fluorescence intensity than nonmutant or single-mutant control cells and reached a higher maximum fluorescence than did single mutants. We sorted cells that had highest fluorescence (**Fig. 2c**), plated them on YPD to form single colonies and genotyped colonies via the markers for each of the deletion loci (*HphMX4* and *URA3*). Based on this test, 15 of 60

unsorted cells were double-mutant, whereas 55 of 60 sorted cells were double-mutant. We also confirmed function of the other diploid- or haploid-selection marker.

Generation of ProMonsters. We transformed MAT**a** *KanMX4*-deletion strains with a universal *GFP* cassette, selected transformants via the *URA3* marker and confirmed integration by PCR. We then individually mated each *GFP*-marked strain with two MATα strains, one carrying *GMToolkit*-**a** and the other carrying *GMToolkit*-α. Mated diploids were selected for Ura^+ and resistance to G418 or Nat resistance conferred by *GMToolkit*-**a** or *GMToolkit*-α, respectively. After sporulation, we established MAT**a** or MATα haploid ProMonster strains carrying both a *GFP* deletion and a *GMToolkit* by Ura^+ and His^+ or Leu^+ selections, respectively.

Genotyping. We designed 16 ABC transporter locus–specific PCR primers from the 5′ flanking sequences and paired each with a common primer complementary to sequence in the *GFP* cassette. PCRs were multiplexed such that genotyping each isolated colony required eight PCRs. We lysed cells in a 96-well format in 2 µl of buffer (0.1 M sodium phosphate (pH 7.4) and 1 unit zymolyase (ZymoResearch)) overlaid with mineral oil, incubating them at 37 °C for 20 min and then at 95 °C for 5 min. The Biomek FX robot (Beckman Coulter) carried out arraying of eight PCR master mixes differing in the primers onto a 384-well plate and addition of the cell lysates to these mixes. PCR products were analyzed using the Gel XL Ultra V-2 electrophoresis system (Labnet) compatible with sample loading using multichannel pipettes.

To gauge the genetic diversity of pools in the *en masse* process, we genotyped lysates from diploid selection mixtures. In all three experimental series, we did not detect deletions in *YNR070W*, *VMR1* and *PDR5* by the third round (**Supplementary Table 11**).

Sexual cycling of green monsters. We cultured sorted GFP-positive cells (2,000 cells) at 30 °C for 48 h in 100 µl of SC-His (MAT**a**) or SC-Leu (MATα) depending on mating type. For mating, we combined MAT**a** and MATα cell cultures 1:1 in <50 µl YPD at 1 $OD_{600\ nm}$, centrifuged the mixture at 735*g* for 5 min and incubated it at 30 °C for 24 h. For processes with isolated and genotyped strains, >28% of haploid cells successfully mated with cells of the opposite mating type ($n \geq 282$ cells). For the *en masse* processes, mating efficiency can be derived from data in **Supplementary Table 5**. We transferred 10 µl of the mating mixture to 500 µl GNA medium (starting $OD_{600\ nm} \cong 0.1$) containing G418 and Nat, and cultured it for 24 h. This allowed for the selection of diploids (**Supplementary Table 5**) owing to resistance to G418 and Nat conferred by *GMToolkit*-**a** (*KanMX4*) and *GMToolkit*-α (*NatMX4*), respectively. We transferred 20 µl of the 1-d culture to 500 µl of fresh GNA medium containing G418 and Nat (starting $OD_{600\ nm}$ was ~0.2) and cultured for 5–7 h to bring cells to the log phase ($OD_{600\ nm}$ was ~1) before sporulation. At this point, we rinsed cells three times with 500 µl of minimum sporulation medium[31] and resuspended them in 1 ml of minimum sporulation medium containing 7.5 µg ml^{-1} lysine, 7.5 µg ml^{-1} leucine, 5 µg ml^{-1} histidine and 5 µg ml^{-1} methionine (to meet auxotrophic requirements). For the *en masse* experiments, we also added 1.25 µg ml^{-1} uracil to the sporulation medium. We evaluated the composition of haploids and diploids in selected pools in the *en masse* process by proxy, using the fact that only diploids should carry both GMTookits and thus be both G418-resistant and Nat-resistant. The sporulation mixture was rotated at room temperature (20–25 °C) for 1 d and then at 30 °C for 3 d[31]. The sporulation efficiency was 5–30%, only counting four-spore asci. We treated 125 µl of this mixture with zymolyase (2 units) at 30 °C in a 50-µl reaction (100 mM sodium phosphate buffer (pH 7.4) and 1 M sorbitol) for 1 h, followed by 5 min of treating with NP-40 (added to the zymolyase reaction to achieve a final concentration of 0.01%), and stopped the zymolyase reaction by adding 500 µl of water and placing it on ice. This mixture was sonicated at the output setting of 1 for one minute with the 50% duty cycle using Sonifier 450 (Branson), split equally into two tubes and centrifuged at 735*g* for 5 min. We confirmed isolated spores by microscopy. To the pellet, we added 100 µl of SC without histidine (SC –His) or SC –Leu to separately select for haploid cells of MAT**a** or MATα, respectively, for 16 to 24 h, using the haploid selection markers (*STE2pr-Sp-his5* and *STE3pr-LEU2*) in the *GMToolkits* (the final $OD_{600\ nm}$ was <0.5). For induction of GFP, we added 100 µl of fresh SC –His (MAT**a**) or SC –Leu (MATα) medium containing doxycycline to the 1-d culture and induced the cells to express GFP for 2 d at 30 °C. The final concentration of doxycycline was 10 µg ml^{-1} unless otherwise noted. We filtered 25 µl of the GFP-induced culture using the cell strainer (BD Falcon) into 1 ml of prefiltered TE buffer (pH 7.5) containing the same concentration of doxycycline, followed by vortexing of the sample before cell sorting.

Fluorescence-mediated cell sorting. We performed cell sorting on a Beckman-Coulter MoFlo cell sorter or a BD Aria sorter equipped with a 488-nm (blue) laser for GFP (FL1) detection. We used gating to avoid cell aggregates, which could exhibit high GFP intensity. The first gate discarded outliers with disproportionately large pulse width, using a plot of pulse width and forward scatter. The second gate selected only cells in the lower 20% in forward scatter (while avoiding the lowest forward and side scatter regions where cell debris is often found), as we expected large forward scatter for cell aggregates. Side scatter and GFP signal were positively correlated. Because simply taking the brightest cells given the gates above would also enrich for cells with a high side-scatter value, the third gate captured approximately the same fraction of cells with the highest fluorescence separately from a series of nonoverlapping ranges of side-scatter value. The selected side scatter ranges were centered around the mode. We checked the sorted (bright GFP) population regularly to confirm that they were consistent with the population mode in terms of forward scatter and side scatter and thus not likely to be rare debris or clumped cells. The number of colonies obtained after plating was consistent with the number of cells sorted.

Microscopy. We used a Nikon Ti inverted microscope equipped with differential interference contrast (DIC) optics with a 100× 1.4-numerical aperture (NA) Plan Apo DIC objective, Hamamatsu Orca-R2 cooled charge-coupled device (CCD) camera, Prior LumenPro fluorescence illuminator and Nikon FTIC-HQ filter set (460–500-nm excitation, 505-nm long-pass dichromatic mirror and 515–560-nm emission). We acquired 12-bit images and converted them to 8-bit images using MetaMorph (version 7.0), and adjusted brightness and contrast and added pseudo-color in an identical manner for all images using Photoshop (Adobe).

doi:10.1038/nmeth.1550

Comparison of time required by techniques for multideletion strain construction. We routinely carried out each round of the green monster process within 13 d, including mating (1 d), diploid selection (1 d), sporulation (4 d), haploid selection (1 d), GFP induction (2 d), growth after flow cytometry sorting (2 d) and genotyping (1–2 d). Multiple processes can be carried out in parallel. Each round of the *en masse* process took 11 d, not requiring genotyping between cycles.

Each round of the *hisG*[11] or Cre-*lox*[14,15] method takes 14 d, including overnight culture before transformation for gene deletion (1 d), colony formation on a selective medium after transformation (3 d), isolation of transformants from background cells by streaking on a fresh plate (2 d), genotyping (1 d), excision of the transformation marker (for example, *URA3*) followed by culture in the presence of a counterselective agent, for example, 5-fluoroorotic acid (4 d), isolation of strains lacking the marker from background cells by streaking on a fresh plate (2 d), and genotyping (1 d). The *delitto perfetto* method[17] needs one extra day (15 d total) per cycle compared to the other 'sequential' methods because marker excision via transformation requires 5 d, including overnight culture (1 d) and transformation followed by counterselection of the transformation marker (4 d).

We generated ABC16 monsters in 11 rounds of the green monster process, consistent with a theoretical total span of 143 d (13 d × 11 rounds). Other methods add deletions sequentially so that deleting 16 genes takes 16 rounds with a total of 224 d (14 d × 16 rounds) for *hisG* or Cre-*lox* and 240 d (15 d × 16 rounds) for *delitto perfetto*, assuming no complications.

Successful *hisG*, Cre-*lox* or *delitto perfetto* process would require genotyping of colonies (for example, using PCR) twice per cycle, for confirming correct gene replacement with a marker and for confirming correct excision of the marker. The *en masse* green monster process does not require genotyping of colonies in parallel, but we recommend this at least to track progress. Genotyping is required if genotyped strains are chosen for subsequent crosses.

Confirmation of genomic integrity of multiple ABC16-monster strain isolates. To assess whether ectopic recombination events had occurred between different *GFP* cassettes, we evaluated four ABC16-monster isolates generated using slightly different construction paths (**Supplementary Fig. 8**). We ran pulse-field gel electrophoresis according to the manufacturer's manual for the CHEF-DR III system (Bio-Rad). For PCR experiments, we used a pair of primers targeting the upstream and downstream genomic regions of each ABC transporter locus to amplify the entire *GFP* cassette (**Supplementary Fig. 4**). Nonmutant (with a *GFP* cassette replacing the *HO* gene) and all 16 single-mutant ProMonster strains served as controls. We used *Taq* polymerase for all experiments except for analysis of the *yll048cΔ* or *ypl058cΔ* locus. Because PCR with *Taq* did not generate bands of the expected size even in the corresponding ProMonster strains, for experiments involving either *yll048cΔ* or *ypl058cΔ* loci, we used Ex Taq (Takara).

To study recombination between *GFP* sequences of nearby genes, we generated a heterozygous double mutant diploid of the genotype *ydl227cΔ*+/+ *ydl242wΔ* and tested in triplicate four DNA samples each representing a mixture of haploid progeny derived from ~30,000 independent meioses of this diploid strain. Two of the triplicates are shown in **Supplementary Figure 5**. We used the four possible combinations for primers upstream and downstream of the open reading frames of the genes *YDL227C* and *YDL242W* to detect any rearrangement.

Statistical analysis. We assessed the impact of fluorescence-based cell-sorting by genotyping isolated clones from either sorted or unsorted cell populations in two experiments: (i) enriching for strains with a large number of deletions derived from a meiotic mixture of a strain heterozygous for all 16 deletions and (ii) enriching for multideletion strains in parallel applications of the *en masse* green monster processes. In the former experiment, we used one-tailed Mann-Whitney *U*-tests (which make no distributional assumptions) to calculate the significance of differences in distributions of numbers of deleted genes in the two sorted samples versus the unsorted control ($n = 24$). In the latter experiment, we used one-tailed tests to compare the sorted samples versus the unsorted samples (n values were 21–34) and two-tailed tests to analyze the differences in distributions of deletion numbers in the sorted and unsorted samples of the current round versus the sorted samples in the previous round (n values were 21–34). All statistical tests used $\alpha = 0.05$.

Drug resistance or sensitivity assays. We started 100-µl cultures using 96-well plates at $OD_{595\,nm} \cong 0.02$ in YPD medium (supporting both fermentation and respiration) with the drug present and continually shook them at 30 °C for 24–48 h with OD_{595} measurements at 5- or 15-min intervals, using an Infinite F200 incubated plate-reading spectrophotometer (Tecan).

For drugs previously used to characterize ABC transporters[7], we assayed strains RY0566 and RY0512 as well as 16 single-mutant ProMonster strains (all MAT**a**). The solvent for all drugs (itraconazole, fluconazole, miconazole, tamoxifen, naftifine hydrochloride, ketoconazole, valinomycin and CCCP from Sigma-Aldrich prepared as 1,000× solutions) was dimethyl sulfoxide (DMSO). Using eight dosage points (**Supplementary Table 12**) in two rounds of drug assay, we determined a small range including IC_{50} for each drug-strain combination. We determined growth curve parameters and calculated IC_{50} values (by linear interpolation) using Matlab (Mathworks) (code freely available upon request).

To assess the drug-sensitivity of the ABC16-monster strain (RY0513; MAT**a**) relative to wild type (BY4742; MATα) or the AD strain[5], we diluted the stock solution from the NIH Clinical Collection 100-fold in YPD for each drug. The ratios of exponential growth rates with drug and without drug (DMSO) between strains served as measures of sensitivity for each drug (**Supplementary Tables 7** and **8**).

PCR primers. Primer sequences are listed in **Supplementary Table 13**.

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