Knocking out multi-gene redundancies via cycles of sexual assortment and fluorescence selection

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Abstract

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, where an important shared function may only be revealed by mutating all family members. Here we describe the 'Green Monster' technology enabling the precise deletion of many genes. In this method, a population of deletion strains with each deletion marked by an inducible green fluorescent protein

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(*GFP*) reporter gene, is subjected to repeated rounds of mating, meiosis, and flow-cytometric enrichment. This results in the aggregation of multiple deletion loci within 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 demonstrate the technology, we generated a single broadly drug-sensitive strain of *Saccharomyces cerevisiae* bearing precise deletions of all 16 adenosine triphosphate-binding cassette transporters within clades associated with multi-drug resistance.

Introduction

Robustness of biological systems to genetic insults is widespread. Synergistic sick or lethal interactions are observed for over 75% of non-essential genes in *Saccharomyces 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 multi-gene interaction, but few of the 80 yeast gene families with six or more genes (Supplementary Table 1 online)[4] have been entirely deleted to identify important underlying shared functions.

Functional redundancy complicates pharmaceutical development. For example, adenosine triphosphate-binding cassette (ABC) transporters overlap in the drugs they export. To isolate the activities of individual transporters, a yeast strain (AD12345678; hereafter termed "AD") was 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 a total of 16 genes[8],[9], motivating further deletions within this family.

In yeast, gene deletion is accomplished by replacement with a selectable marker via homologous recombination[10]. However, engineering strains bearing many unlinked deletions presents challenges: (1) If deletions are sequentially introduced, the time required scales linearly with the number of target genes. (2) 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. (3) Finally, there are a limited number of useful selectable markers.

All commonly used methods suffer from the first two problems, but some have addressed the third by removing markers before re-use. 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 knock-out fragments[12],[13]. The Cre/*lox* method improves marker excision efficiency and leaves a shorter remnant sequence[14],[15]. However, Cre recombinase also catalyzes massive genome rearrangements between non-adjacent *loxP* sites (E. Boles, personal communication [15],[16]. 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 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 multi-drug 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 the green fluorescent protein (*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 individual loci can be engineered and marked to allow selection or efficient screening on the basis of gene dosage, and alleles can be reassorted via mating or its equivalent (such as viral transfection or bacterial conjugation). Here we implement the strategy for the yeast *S. cerevisiae*.

The initial ingredient is a set of 'ProMonster' strains, each carrying an identical inducible *GFP* reporter replacing one of the target genes ('your favorite genes' or *YFG*s; Fig. 1b). To facilitate repeated sexual assortment of these deletions, ProMonsters of mating types (*MAT*s) **a** and α carry the GMToolkit-**a** and GMToolkit-α constructs, respectively, at the *CAN1* locus (Fig. 1c). Each GMToolkit contains: (1) 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 *MAT*α-specific *STE3* promoter[19]; (2) one of two drug-selectable markers—either *KanMX4* conferring resistance to G418 (G418^R) or *NatMX4* conferring resistance to nourseothricin (Nat^R)[20]— collectively allowing selection for *MAT***a**/*MAT*α diploid cells carrying both GMToolkits, and (3) the Tet activator *rtTA*[21] for *GFP* induction enabling 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

GFP intensity varies even within 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 ProMonsters of each mating type yielded a population with 99% of cells deleted in all 24 genes in 12 rounds (Fig. 2a). It may seem counterintuitive that cells carrying 24 *GFP* copies could be separated from cells carrying 23 *GFP* copies when GFP expression at each locus can vary amongst cells by 50%; however, the extreme high-intensity tail of the distribution should be dominated by the cells carrying the most *GFP* copies.

To combine deletions on the same chromosome, a meiotic crossover must occur between deleted loci. When simulating an extreme case where all deletions are on one chromosome spaced ~15 kb apart, the final multi-mutant dominated the population in 16 rounds (Fig. 2b),

as opposed to 12 rounds if each deletion locus were independently assorting. 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 $tetO_2pr\text{-}GFP$ deletion cassettes (Fig. 1b), as well as GMToolkit-**a** and GMToolkit-α (Fig. 1c). Given the existing library of *KanMX4* deletion strains, these cassettes enable 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 (n = 24). We also generated a cassette with *HphMX4*[20] instead of *URA3*. Should GFP expression saturate the production capacity of cells or become toxic, the $tetO_2$ promoter allows GFP expression to be reduced by lowering doxycycline concentration. For essential target genes, $tetO_2pr\text{-}GFP$ cassettes can be linked to temperature-sensitive[22] or other informative alleles[23].

To assess each component, we mated a *MAT***a** strain carrying the $HphMX4\ tetO_2pr\text{-}GFP$ cassette with *MAT*α cells carrying both a $URA3\ tetO_2pr\text{-}GFP$ cassette at a different unlinked locus and GMToolkit-α (Online Methods). The resulting diploids were then sporulated. Assuming normal meiotic segregation of two unlinked genes, we expected *MAT*α GMToolkit-α cells selected from this meiotic mix to be a 1:2:1 mixture of no-*GFP*, 1-*GFP* and 2-*GFP* cells. We collected the most fluorescent cells (Fig. 2c) and genotyped them using *HphMX4* and *URA3* markers. Unsorted cells were 25% double-mutant (n=60) as expected, whereas sorted cells were 92% double-mutant (n=60), demonstrating selection of multi-mutants using GFP intensity.

Deleting 16 ABC transporter genes

To test the scalability of the Green Monster process, we targeted all 16 genes within the ABCC and ABCG clades in the *S. cerevisiae* ABC transporter family (these clades contain multiple genes associated with multi-drug resistance)[8,9]. For each of the 16 targets, we generated both *MAT***a** and *MAT*α ProMonster strains. When we combined all strains in a pool and treated them with one round of the process (Supplementary Fig. 1 online), 100% (n=12) of the resultant haploids were double mutants (~25% would be expected from random segregation). Genotyped double-mutant strains were crossed—either in pools or individually—with opposite mating-type strains (Supplementary Figs. 1 and 2 online). 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 online). This measure of enrichment is conservative in that expectations did not account for potential under-representation of higher-order mutants due to reduced fitness. Because many crosses can proceed in parallel, the total time required scales not with the total number of crosses, but with the greatest number of rounds in any lineage from ProMonster to the final multi-mutant.

We obtained 'ABC16-monsters' with all 16 transporter genes deleted with no more than 11 rounds along any lineage. Because each round of the Green Monster process requires 13 days (Online Methods), 143 days would thus be sufficient for construction of the ABC16-monsters given 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. While ectopic reciprocal recombination is seen with the Cre/*lox* method (E. Boles, personal communication)[15],[16], the ABC16-monster had no recognizable karyotype defects (Supplementary Fig. 3 online). 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 online; 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 (Fig. 2d) and in micrographs (Fig. 2e). The level of GFP expression in these cells—even with high levels of the inducer doxycycline—did not affect growth rates (Supplementary Table 3 online).

To confirm the effectiveness of flow cytometry in enriching for strains with more than a few deletions, we crossed the ABC16-monster with a non-mutant strain, and genotyped both sorted and unsorted haploid progeny resulting from one round of the Green Monster process. By sorting the 1% brightest cells, we obtained a greater average of 9.6 ± 0.3 (s.e.m.) deletions ($n = 24$ for all samples), as compared with 7.6 ± 0.5 deletions for the unsorted sample ($P = 0.002$). Sorting the top 0.1% of cells achieved an average deletion number of 10.3 ± 0.4 ($P = 0.0002$).

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., unpub. data). We completed the generation of this six-deletion strain in three rounds (Supplementary Table 4 online). 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, whereas PCR designed to detect a cassette-mediated recombination between loci did not detect any rearrangement (Supplementary Fig. 5; Methods).

Evaluating the en masse Green Monster process

The processes described above exploited both GFP fluorescence and genotyping to enrich for multi-mutants. 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 online), such that heterogeneous pools of deletion strains emerging from each fluorescence-based selection were taken directly into the next round and mated 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 online). 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; Supplementary Table 6 online). 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 to 11 days and reduce the number of processes that are run in parallel. It could be widely applicable, particularly where multi-mutant 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], conditional[22] alleles, or by ectopic expression of target genes during strain construction. However, PCR-based genotyping can maintain the presence of alleles with reduced fitness or fluorescence intensity, is not labor-intensive, and reveals progress during the process.

Demonstrating 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 a panel of ~400 drugs previously used in human clinical trials (NIH Clinical Collection; Supplementary Table 7 online). The ABC16-monster exhibited sensitivity to a comparable number of drugs as the AD strain (Fig. 3a; Supplementary Table 8 online). 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 online). Resistance to some drugs, observed for both AD and ABC16-monster strains (e.g., 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 ('multi-gene redundancy'), we compared 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; Methods). At least three patterns of drug response emerge.

In the first pattern, exemplified by tamoxifen and carbonylcyanide *m*-chlorophenylhydrazone (CCCP), the ABC16-monster was more sensitive than non-mutant 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 (e.g., Pdr5p and Pdr11p for tamoxifen and Snq2p for CCCP, based on Fig. 3b), with little compensatory activity provided by the other transporters.

The second drug response pattern is exemplified by fluconazole, ketoconazole, miconazole, and itraconazole. The ABC16-monster was highly sensitive, similarly to the AD strain[7], while single mutants other than *pdr5Δ* did not have marked sensitivity (Fig. 3b, c). This suggests that transporters beyond 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 online).

The third pattern of drug response was represented by valinomycin, an ionophore that disrupts mitochondrial function. The ABC16-monster was unexpectedly more resistant than non-mutant or any single mutant to valinomycin (Fig. 3c). Since thirteen single mutants had slight resistance compared to non-mutant cells, the monster may simply have the aggregated effects of single deletions. Previously, increased resistance in ABC-transporter deletion strains was observed for substrates of Pdr5p, Snq2p, and Yor1p[25]. In this previous example, specific efflux pumps responsible for the resistance were already known. 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 shows 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 employed 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.

Illustrating the potential value of this technology, an ABC16-monster strain we constructed is complementary to a previous drug hyper-sensitive strain (AD) in that it reveals 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 were 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 not found in some other technologies for strain construction: it can 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 shares limitations with all other strategies. Where genes are essential (either individually or in combination) the process cannot proceed without the use of hypomorphic, conditional, or ectopically-expressed 'covering' alleles. Like other methods, it is subject to the appearance of adaptive mutations. However, the Green Monster process may be less subject to aneuploidies (present in at least 8% of deletion strains[30]), given that the strains it produces must repeatedly survive meiosis.

The Green Monster process has limits not shared by other methods. 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 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 that 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 can already envision application of the strategy in three important model organisms (Supplementary Fig. 7 online provides an overview in *E. coli*, *C. elegans*, and mouse).

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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS

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a

b

c
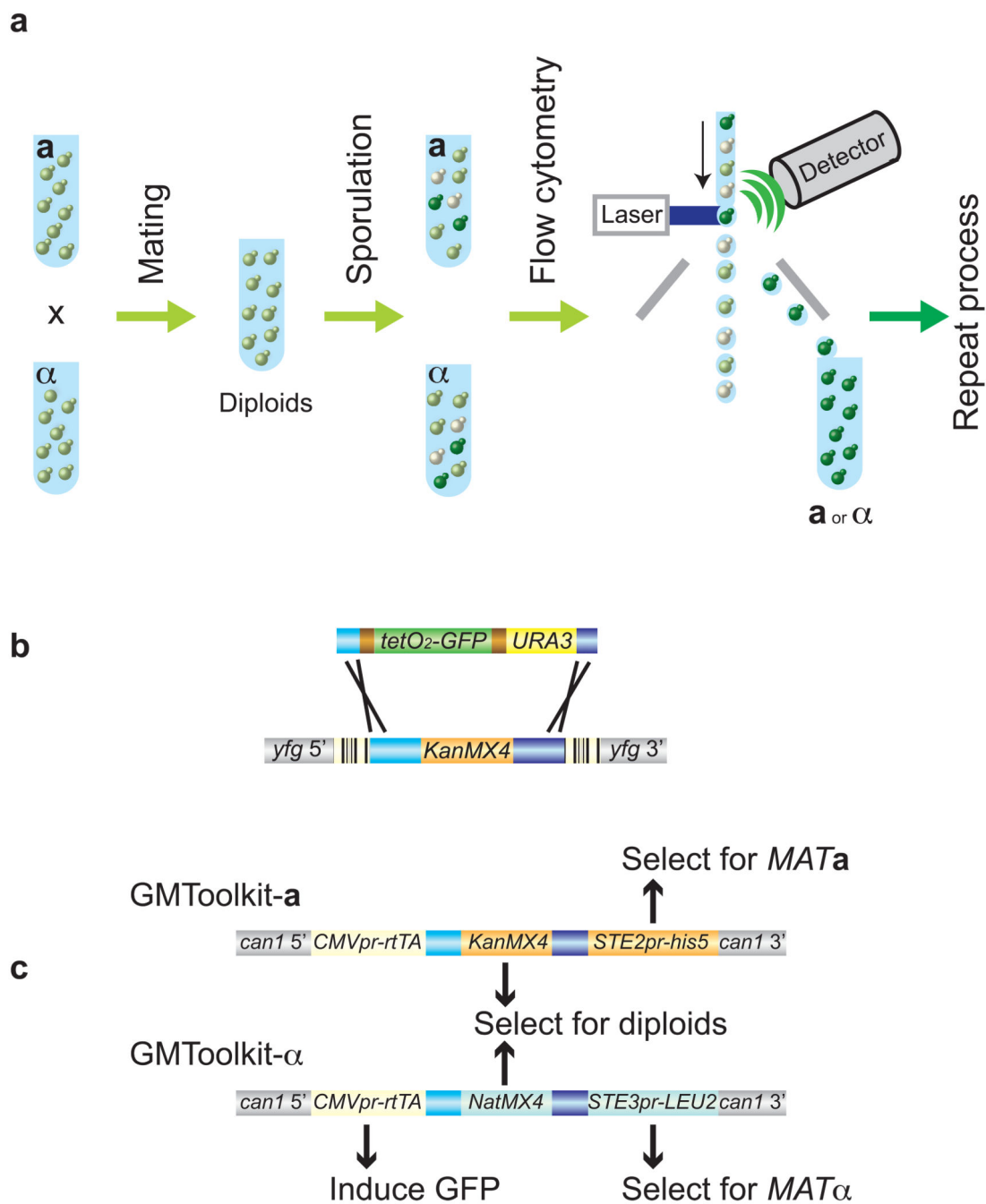
Figure 1.
Design of the Green Monster process. (**a**) Schematic overview of the process In yeast, crossing different haploid single-mutants generates 0-deletion (off-white), 1-deletion (light green), and 2-deletion (dark green) cells. From this mixture, flow cytometry enriches for 2-deletion cells. Higher-order multi-mutants are assembled via repeated rounds of sexual assortment and enrichment. (**b**) Universal *GFP* deletion cassette replaces *KanMX4* in 'your favorite gene' (*YFG*) via recombination within *TEF* subsequences, while maintaining barcodes (stripes) intact. The inducible *tetO$_2$* promoter allows titration of GFP expression.
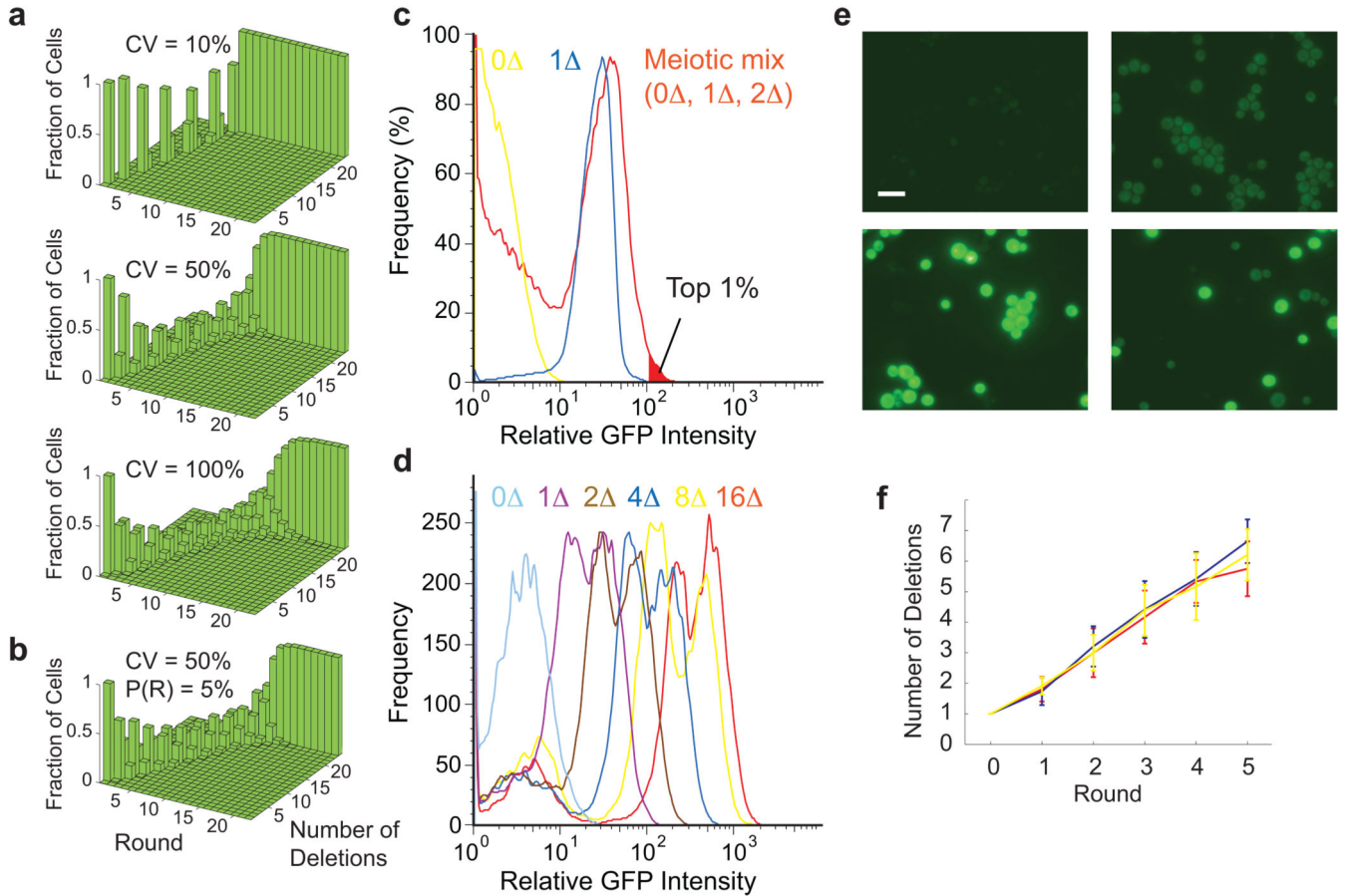
Transcriptional terminators (brown) and the *TEF* promoter (light blue) and terminator (dark blue) are shown. Selectable marker: *URA3*.. (**c**) GMToolkits, inserted at the *CAN1* locus, contain rtTA[21], either *KanMX4* and *STE2pr-Sp-his5* (GMToolkit-**a**) or *NatMX4* [20] and *STE3pr-LEU2* [19] (GMToolkit-α).
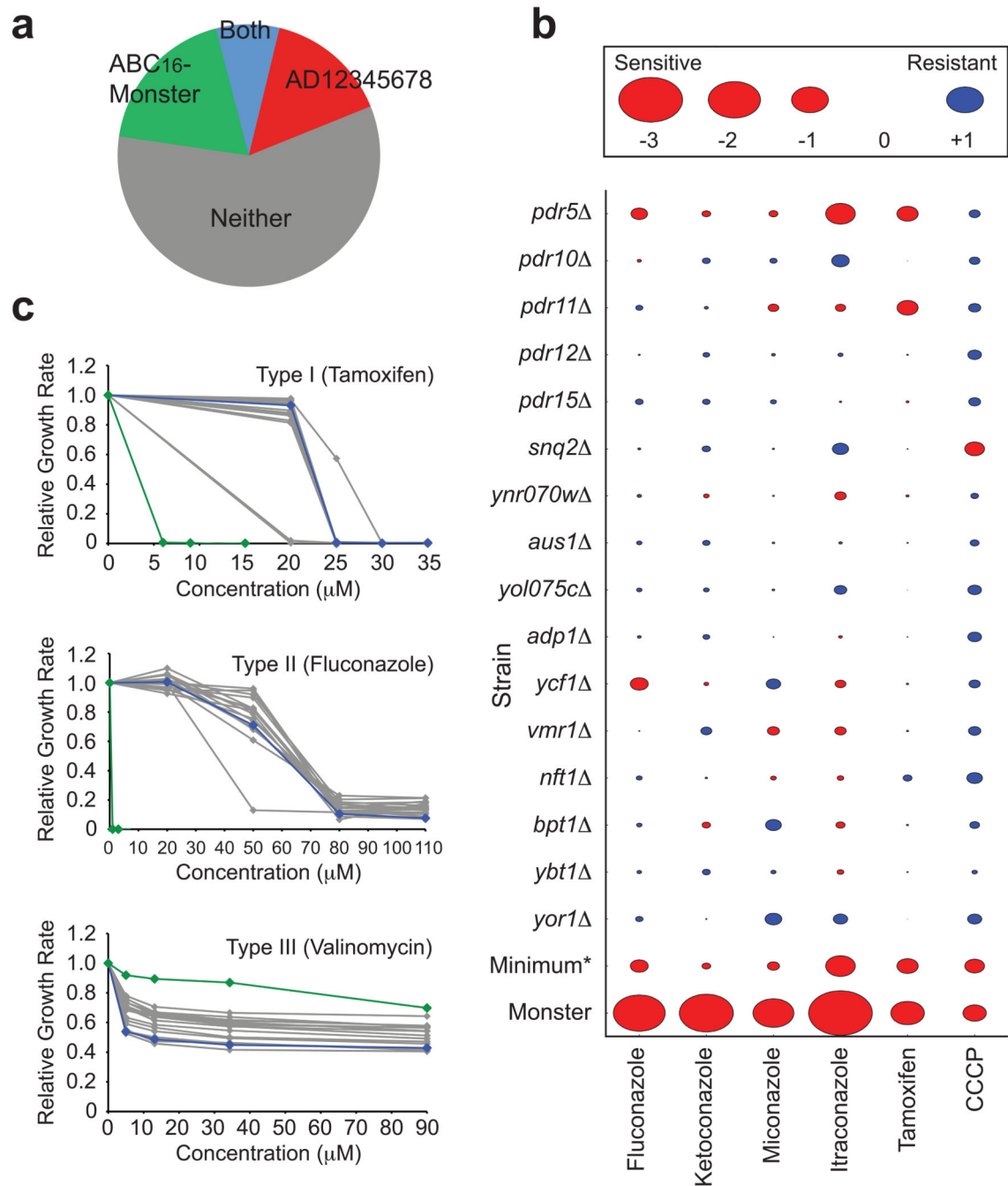
Figure 2.
Demonstration of the Green Monster process. (**a**) Simulations showing that >99% of a cell population accumulate all 24 deletions in eight (top), 12 (middle), or 19 rounds (bottom), with greater efficiency for lower coefficient of variation (CV) of GFP intensity (achievable using internal standard to control for noise (**b**) Simulation showing that 24 linked deletions with the meiotic crossover probability between adjacent loci (P(R)) of 5% can assemble in 16 rounds when the GFP CV is 50%. (**c**) Cell sorting strategy. Histograms (y-axis: the percentage of maximum cell number) are shown for GFP intensity in no-GFP cells (0), single-GFP cells (1), 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 cells. The brightest 1% of the cells in the meiotic mix were collected (red filled area). (**d**) GFP intensity of multi-mutants. 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 non-mutant (top left), double-mutant (top right), the ABC16-monster (bottom left), and a mixture of double mutant and ABC16-monster (bottom right) cells. Identical exposure, brightness, and contrast settings were used for images. Scale bar indicates 10 μm. (**f**) The en masse Green Monster process. Average deletion numbers for each round from three independent processes are plotted (red, blue, and yellow). Error bars indicate standard deviations (n = 21 – 34).

Figure 3.
Hypersensitivity of the ABC16-monster to drugs. (**a**) Pie chart showing the number of drugs to which the ABC16-monster or the previously-described drug-hypersensitive AD strain is more sensitive. (**b**) A comparison of ABC16-monster and single-mutant drug sensitivity. The IC_{50} for each strain/drug combination relative to that of the corresponding non-mutant/drug combination was calculated. Area of each shape is proportional to this relative IC_{50} value. *: 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), non-mutant (blue), and single-deletion strains (grey) as a function of concentration of tamoxifen, fluconazole, and valinomycin.