ONLINE METHODS

Yeast strains. All yeast strains have the background genotype of BY4741 (*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*) or BY4742 (*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*)[31] except AD12345678 (*MATα 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 share the background *MATα lyp1Δ his3Δ1 leu2Δ0 ura3Δ0 met15Δ0* and carry *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 G418 (Sigma-Aldrich) and 100 μg/ml nourseothricin (Nat; WERNER BioAgents), respectively. The genotypes of ABC16-monsters (RY0512, RY0513, RY0521, etc.) are *MAT***a** (or *MATα*) *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) are *MAT***a** and *MATα* ProMonster strains (see below) containing the GFP deletion cassette in the neutral *ho* deletion. 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 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, with results visualized using MATLAB (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 intensity for each cell by summing independent samples from a Gaussian distribution for each GFP locus, modeling the coefficient of variation (CV) between cells of GFP 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 intensity. We modeled deletion loci as linked or independently assorting (i.e., 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 full-length, wild-type GFP coding sequence amplified with primers containing *Bam*HI and *Apa*I sites into the *Bam*HI and *Apa*I sites of pCM251[32] to make pYOGM002a, cloned a 1.5-kb region containing *ADHterm-tetO*$_2$*-GFP-CYC1term* amplified from pYOGM002a into the *Xho*I and *Hin*dIII sites of pBluescript SK+ (Stratagene) to make pYOGM005, and cloned a 1-kb *Eco*RI-*Xba*I *URA3* PCR fragment into pYOGM005 to make pYOGM007a. We introduced the S65T change in the GFP sequence on pYOGM007a to make pYOGM012. PCR with primer sequences GGATCCCCGGGTTAATTAAGGCGCGCCAGATCTGTTTAGCTTGCCCAAGCTCCTCGAGTAATTCG (Primer A) and GGCGTTAGTATCGAATCGACAGCAGTATAGCGACCAGCATTCACGTACCGGGTAATAACTGATATAAT produced a *KanMX4* replacement fragment. Similarly, we made the *HphMX4*[20] version of the targeting plasmid (pYOGM013). The difference is that, instead of the *URA3* fragment, we cloned a 1.5-kb *HphMX4* PCR fragment from pAG32[20] with *Xba*I and *Not*I sites at the ends into pYOGM005. PCR with Primer A and a primer with the sequence CAGTATAGCGACCAGCATTCAC produced a *KanMX4*-targeting fragment.

Construction of the GMToolkits. For assembling GMToolkit-**a**, we cloned a *KanMX4* PCR fragment flanked by *Xba*I and *Xho*I sites into *Xba*I and *Sal*I sites of pUC18 to make pYOGM014a, and cloned a *CMVpr-rtTA*[33,34] PCR fragment flanked by *Kpn*I and *Nhe*I sites into the *Kpn*I and *Xba*I 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*[19] PCR fragment flanked by *Bam*HI and *Apa*I sites into *Bam*HI and *Apa*I sites of pBluescript SK+ to make pYOGM015a, cloned a *NatMX4*[20] PCR fragment flanked by *Xba*I and *Xho*I sites into *Xba*I and *Sal*I sites of pYOGM015a to make pYOGM018a, and cloned a *CMVpr-rtTA* PCR fragment flanked by *Nhe*I sites into the *Xba*I 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 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 *YJL187C*—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; Calbiochem)[20]. Some integrants were crossed with a *MAT*α GMToolkit strain, using the method described in Generation of ProMonsters (see below). For confirming functions of molecular tools, we mated *MAT***a** cells [*yjl187cΔ::HphMX4 tetO*$_2$*pr-GFP*] with *MAT*α cells [*snq2Δ::URA3 tetO*$_2$ *pr-GFP can1Δ::*GMToolkit-α]. From this cross, we selected diploids using *HphMX4*[20] and *NatMX4* (in GMToolkit-α). After sporulation of diploids (see Sexual cycling of Green Monsters below), we selected *MAT*α cells using SC-Leu medium by virtue of the GMToolkit-α marker *STE3pr-LEU2*, which is activated only in *MAT*α 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*, 1-*GFP*, and 2-*GFP* cells, respectively. When analyzed using flow cytometry (**Fig. 2c**), the mixture of cells had a wider distribution of fluorescence intensity than non-mutant or single-mutant control cells and reached a higher maximum fluorescence than single mutants. We sorted cells that were highest in 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 out of 60 unsorted cells were double-mutant, whereas 55 out 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 successful integration by PCR. We then individually mated each GFP-marked strain with two *MAT*α strains, one carrying GMToolkit-**a** and the other carrying GMToolkit-α. Mated diploids were selected for Ura$^+$, and G418^R or Nat^R conferred by GMToolkit-**a** or GMToolkit-α, respectively. Following sporulation, we established *MAT***a** or *MAT*α 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. PCR reactions were multiplexed such that genotyping each isolated colony required eight PCR reactions. We lysed cells in a 96-well format in 2 μl of buffer [0.1 M sodium phosphate (pH 7.4), 1 unit zymolyase (ZymoResearch)] overlaid with mineral oil, incubating them at 37°C for 20 minutes and then at 95°C for 5 minutes. 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 multi-channel pipettes.

To gauge the genetic diversity of pools in the *en masse* process (see below), 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** online).

Sexual cycling of Green Monsters. We cultured sorted GFP-positive cells (n > 2,000) at 30°C for 48 hours in 100 μl of SC-His (*MAT***a**) or SC-Leu (*MAT*α) depending on mating type. For mating, we combined *MAT***a** and *MAT*α cultures 1:1 in <50-μl YPD at 1 OD_{600}, centrifuged the mixture at 735 *g* for 5 minutes, and incubated it at 30°C for 24 hours. For processes with isolated and genotyped strains, >28% of haploid cells mated with cells of the opposite mating type ($n \geq 282$). For the *en masse* processes, mating efficiency can be derived from data in **Supplementary Table 5** online. We transferred 10 μl of the mating mixture to 500 μl GNA medium (starting $OD_{600} \cong 0.1$) containing G418 and Nat, and cultured it for 24 hours. This allowed for the selection of diploids (**Supplementary Table 5** online) due to $G418^R$ and Nat^R conferred by GMToolkit-**a** (*KanMX4*) and GMToolkit-α (*NatMX4*), respectively. 20 μl of the one-day culture was transferred to 500 μl of fresh GNA containing G418 and Nat (starting OD was ~0.2) and cultured for five to seven hours to bring cells to the log phase (OD 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 lysine, 7.5 μg/ml leucine, 5 μg/ml histidine, and 5 μg/ml methionine (to meet auxotrophic requirements). For the *en masse* experiments, we also added 1.25 μg/ml 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 $G418^R$ and Nat^R. The sporulation mixture was rotated at room temperature for one day and then at 30°C for three days[31]. The sporulation efficiency was between 5% and 30%, only counting four-spore asci. We treated 125 μl of this mixture with zymolyase (2 units; ZymoResearch) at 30°C in a 50-μl reaction (100 mM sodium phosphate buffer, pH 7.4 and 1 M sorbitol) for 1 hour, followed by five minutes 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 them 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 minutes. We microscopically confirmed isolated spores. To the pellet, we added 100 μl of SC-His or SC-Leu to separately select for haploid cells of *MAT***a** or *MAT*α, respectively, for 16 to 24 hours, using the haploid selection markers (*STE2pr-Sp-his5* and *STE3pr-LEU2*) within the GMToolkits (the final OD was <0.5). For induction of

GFP, we added 100 µl of fresh SC-His (*MAT***a**) or SC-Leu (*MAT*α) medium containing doxycycline to the one-day culture and induced the cells to express GFP for two days at 30°C. The final concentration of doxycycline was 10 µg/ml unless otherwise noted. We filtered 25 µl of the GFP-induced culture using the cell strainer (BD Falcon) into 1 ml of pre-filtered 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), since 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 level of fluorescence separately from a series of non-overlapping 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 DIC optics with a 100× 1.4-NA Plan Apo DIC objective, Hamamatsu Orca-R2 cooled 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.

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

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

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

Successful *hisG*, Cre/*lox*, or delitto perfetto process would require genotyping of colonies (e.g., 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** online). 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** online). Non-mutant (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 where the *yll048cΔ* or *ypl058cΔ* locus was analyzed. 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 Fig. 5** online. 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: (1) enriching for strains with a large number of deletions derived from a meiotic mixture of a strain heterozygous for all 16 deletions and (2) enriching for multi-deletion 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 vs. the unsorted control ($n = 24$). In the latter experiment, we used one-tailed tests to compare the sorted samples vs. the unsorted samples (n ranged from 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 vs. the sorted samples in the previous round (n ranged from 21-34). All statistical tests used $\alpha = 0.05$.

Drug resistance/sensitivity assays. We started 100-μl cultures using 96-well plates at $OD_{595} \cong 0.02$ in YPD medium (supporting both fermentation and respiration) with drug, and continually

shaken them at 30°C for 24 - 48 hours with OD_{595} measurements at 5- or 15-minute intervals, using an Infinite F200 incubated plate-reading spectrophotometer (Tecan).

For drugs previously used to characterize ABC transporters[7], we assayed RY0566, RY0512, and 16 single-mutant ProMonsters (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 (listed in **Supplementary Table 12** online) 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 (code freely available upon request).

To assess the drug-sensitivity of the ABC16-monster strain (RY0513; *MAT***a**) relative to wild type (BY4742; *MAT*α) 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** online).

PCR primers. Supplementary Table 13 online lists primer sequences.

Supplementary File	Title
Supplementary Figure 1	The early phase of ABC16-monster construction
Supplementary Figure 2	The later phase of ABC16-monster construction
Supplementary Figure 3	Karyotypes of ABC16-monster and wild-type strains
Supplementary Figure 4	Evidence for lack of recombination between GFP cassettes within four ABC16-monster isolates
Supplementary Figure 5	Absence of GFP-mediated rearrangements between two nearby cassettes
Supplementary Figure 6	Schematic diagram of one implementation of the *en masse* variant of the Green Monster process
Supplementary Figure 7	Schemes for applying the Green Monster process in other species
Supplementary Figure 8	Later part of the lineage of the ABC16-monsters
Supplementary Table 1	Common Interpro entries in *S. cerevisiae*
Supplementary Table 2	Enrichment for deletions in the lineage leading to the ABC16-monsters
Supplementary Table 3	Growth of ABC16-monsters in the presence of the inducer doxycycline
Supplementary Table 4	Deletion numbers of strains during a Green Monster process with six loci
Supplementary Table 5	Composition of populations from the *en masse* Green Monster processes
Supplementary Table 6	Statistical analysis of the *en masse* processes
Supplementary Table 7	Cumulative frequency of drugs for each drug-affected growth rate range
Supplementary Table 8	Drug assay using drugs of the NIH Clinical Collection
Supplementary Table 9	IC50 value for each strain-drug combination (μM)
Supplementary Table 10	Relative fitness values for the ABC16-monster and the multiplicative model based on single mutant relative fitness
Supplementary Table 11	Deletions not detected in diploid-selection mixtures or isolated and genotyped sorted cells in the *en masse* Green Monster processes
Supplementary Table 12	Drug concentrations used to characterize *ho* non-mutant control, the ABC16-monster (*MAT* **a**), and sixteen single mutant strains
Supplementary Table 13	List of primers