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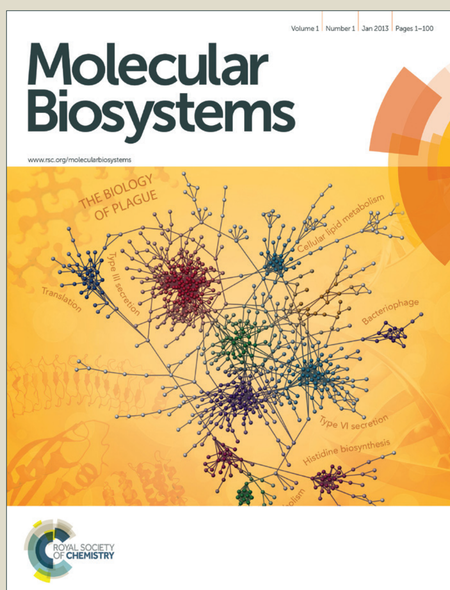

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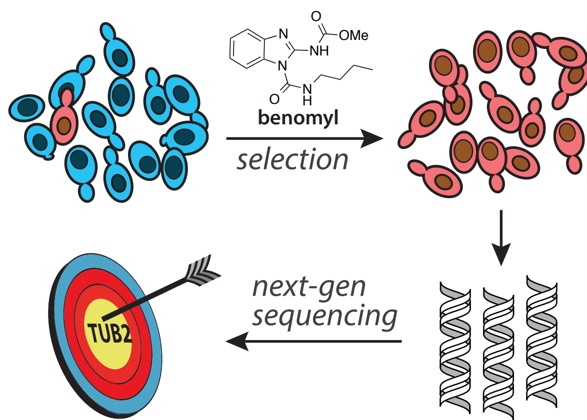

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Confirmation of the cellular targets of benomyl and rapamycin using next-generation sequencing of resistant mutants in *S. cerevisiae*

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Abstract

Investigating the mechanisms of action (MOAs) of bioactive compounds and the deconvolution of their cellular targets is an important and challenging undertaking. Drug resistance in model organisms such as *S. cerevisiae* has long been a means for discovering drug targets and MOAs. Strains are selected for resistance to a drug of interest, and the resistance mutations can often be mapped to the drug's molecular target using classical genetic techniques. Here we demonstrate the use of next generation sequencing (NGS) to identify mutations that confer resistance to two well-characterized drugs, benomyl and rapamycin. Applying NGS to pools of drug-resistant mutants, we develop a simple system for ranking single nucleotide polymorphisms (SNPs) based on their prevalence in the pool, and for ranking genes based on the number of SNPs that they contain. We clearly identified the known targets of benomyl (*TUB2*) and rapamycin (*FPR1*) as the highest-ranking genes under this system. The highest-ranking SNPs corresponded to specific amino acid changes that are known to confer resistance to these drugs. We also found that by screening in a *pdr1Δ* null background strain that lacks a transcription factor regulating the expression of drug efflux pumps, and by pre-screening mutants in a panel of unrelated anti-fungal agents, we were able to mitigate against the selection of multi-drug resistance (MDR) mutants. We call our approach "Mutagenesis to Uncover Targets by deep

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Sequencing, or "MUTseq", and show through this proof-of-concept study its potential utility in characterizing MOAs and targets of novel compounds.[a]

Introduction

Phenotypic screening provides a powerful mechanism for identifying compounds with novel mechanisms of action (MOAs). Such compounds can become therapeutic leads themselves, or can be used to illuminate new druggable targets. According to one survey, of the 50 first-in-class small molecule drug approvals with novel molecular MOAs from 1999-2008, 28 were discovered through phenotypic screens while only 17 were discovered from target-based programs[1]. The major drawback with phenotypic approaches is that there is no general method for identifying the molecular targets of active compounds. The most common approaches to target ID have involved biochemical purification and affinity-based methods, which often require the costly and time-consuming synthesis of covalently immobilized derivatives. Notable exceptions include the recent application of deep sequencing technology to pinpoint drug resistance mutations in HCT-116 cells[2] and the use of mass spectrometry to identify targets based on their stability to proteolysis in the presence of ligand[3].

Although relatively uncommon in higher eukaryotes, genetic methods have been used for some time to facilitate target ID in simpler systems, especially fungi and bacteria. The budding yeast *S. cerevisiae,* in particular, is an excellent model system for the study of the mechanisms of action of small molecules due to the relative ease with which it can be manipulated genetically and the high degree of conservation in basic cellular processes between yeast and higher eukaryotes (reviewed in[4]). Indeed, a variety of genome-wide tools have been developed for investigating small molecule MOAs in *S. cerevisiae*. These include the use of barcoded deletion strains to identify chemical-genetic interactions[5-8], high-copy expression libraries to identify phenotypic suppressors[9-11], and high-throughput complementation strategies using heterologous expression of barcoded open reading

[a] In a previously published paper[41] the term "Mut-seq was used to describe a method to probe essential amino acids in the T7 bacteriophage genome. In contrast, our method, "MUTseq", is an acronym to describe our method, "Mutagenesis to Uncover Targets by deep Sequencing".

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frames(ORFs)[12]. More recently, an ultra-diverse, barcoded "variomic" library containing thousands of alternate alleles for every yeast gene was used to identify drug resistance alleles which can point directly to drug targets[13]. Using these strategies, not only primary targets, but also "off-target" activities and alternate modes of action for a number of drugs have been identified in yeast[14].

In contrast to techniques that rely on genomic libraries of yeast strains and expression constructs, point mutations that confer drug resistance can be mapped directly to a drug's molecular target. This strategy was used, for example, to identify the targets of rapamycin, TOR1 and TOR2 in yeast[15-17], which ultimately helped to confirm the important homolog mTOR in humans[18]. In addition, targets of the antifungal compounds LY214352 (dihydroorotate dehydrogenase) [19] and UK-118005 (RNA Pol III)[20] were identified by cloning drug resistance genes. In general, classical genetic techniques are employed to characterize specific drug resistance mutations in yeast[20] [21]. These methods require genetic crosses and the cloning of large numbers of mutant alleles of genes, and also a plentiful supply of compound, which, in many cases, may be in limited supply and/or difficult to synthesize.

Next-generation sequencing (NGS) technology has made whole-genome sequencing a viable alternative to traditional genetic mapping approaches. Mutations that confer drug resistance can be pinpointed by simply comparing sequence reads of compound-resistant strains to those of the parental strain. Genes or pathways that display an enrichment in new mutations represent potential targets. For example, the target of a new anti-tuberculosis drug was identified by whole-genome sequencing of resistant clones[22], and NGS approaches have been used to identify mutations responsible for echinocandin resistance in *Candida galbrata*[23]. Deep sequencing was also used to identify mutations that confer resistance to oxidative stress in *S. cerevisiae*[24].

Although these studies point toward whole-genome sequencing as an attractive approach for characterizing drug resistant mutants in *S. cerevisiae*, the specific application of NGS toward the identification of small molecule targets has not been reported. Here we describe the use of NGS to identify drug targets in yeast using a straightforward approach that does not involve the use of tagged genomic libraries or require downstream genetic manipulations. We found that screening for resistance mutants in a *pdr1Δ* deletion strain minimized the selection of multi-drug resistant (MDR) mutants, and

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demonstrate that MDR false positives can be further limited by performing cross-resistance screens of candidate mutants in a panel of unrelated drugs. We identified the known targets of benomyl and rapamycin using this approach (Figure 1), and show that NGS offers an orthogonal technique to other chemical genetic approaches available for studying small molecule MOAs in yeast.

Methods

Resistant Mutant selection. The *pdr1Δ* strain used for this study was created via homologous recombination from the background strain, BY4741, a derivative of S288C[25](see Supplemental Information for genotype). A preliminary growth study was conducted for both benomyl and rapamycin to determine an optimal drug screening concentration. YPD plates containing 1X, 5X, 10X, 20X, and 40X the IC_{50} of each compound (IC_{50} = 30 µM for benomyl; 25 nM for rapamycin) were inoculated with ~10^7 *pdr1Δ* cells and incubated for two days at 30˚C. The lowest concentration at which less than five colonies were observed was chosen as the dose for the selection of resistant mutants. The optimal selection concentrations for benomyl and rapamycin were determined to be 150 µM and 0.25 µM, respectively. To mutagenize cells with ethyl methanesulfonate (EMS), 1 mL of an overnight culture (~10^8 cells/mL) of *pdr1Δ* was added to a 1.5 mL microcentrifuge tube and pelleted by centrifugation. The supernatant was discarded and the pellet was resuspended in sterile water. The cells were pelleted again and then resuspended in 1 mL of 0.1 M sodium phosphate buffer at pH 7. Next, 30 µL of EMS was added to the EMS sample tube and the tube was vortexed for 15 s and then incubated with inversion at 30° C for 1 h. After incubation, the cells were pelleted and resuspended in 200 µL of 5% sodium thiosulfate to quench the remaining EMS, and then transferred to a clean tube. This thiosulfate wash step was repeated for a total of three times. After the final wash the pellet was resuspended in 1 mL of water, plated in 100 µl aliquots (~10^7 cells) onto 10 plates containing the selection dose determined above and incubated at 30° C for 2 days. As a control, a second aliquot of cells treated

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identically except for the omission of EMS was selected for resistance in an attempt to discover spontaneous drug-resistant mutants.

Confirmation of resistance and MDR cross-resistance screening. EMS-treated and spontaneously resistant mutants from the initial selection were confirmed by re-streaking onto YPD/agar plates containing compound, along with the parental starting strain to serve as a non-viable control. Mutants that yielded colonies within 3 days were considered resistant and evaluated further in a multi-drug resistance cross-screen. This screen was performed using the 384 halo assay as previously reported [26]. Overnight cultures of resistant mutants were seeded in YPD top-agar at an OD_{600} of 0.06 and poured into OmniTrays. After the agar solidified, lethal doses of sixteen known anti-fungal compounds (see Supporting Information for a list of the anti-fungals used), dissolved in DMSO, were pinned into the agar. Plates were incubated at 30° C overnight and then analyzed using an optical density plate reader to quantify growth inhibition by assigning each anti-fungal a 'halo score' for that particular resistant strain.

Genomic DNA preparation. Mutants chosen for sequencing were grown overnight in 10 mL YPD liquid at 30°C. To pool samples, cultures of individual mutants were diluted to equal ODs and equal amounts of each strain were mixed to give a final volume of 10 mL. Cells were pelleted by centrifugation, resuspended in 1 mL of sterile water and transferred to a 1.5 mL microcentrifuge tube. The cells were pelleted again and resuspended in 200 µl of lysis buffer (1% SDS, 2% Triton X 100, 100 mM NaCl, 10 mM Tris pH 8, 1 mM EDTA). Approximately 3 g of acid-washed glass beads and 200 µl of phenol:chloroform:isoamyl alcohol (25:24:1) were added to the resuspended cells, which were then vortexed for 3 min. An additional 200 µl of TE was added to the tube and the mixture was centrifuged for 5 min. Following centrifugation, 350 µl of the aqueous (top) layer was carefully removed with a pipette and transferred to a new tube with 1 mL of cold 100% ethanol. The DNA was allowed to precipitate at -20° for at least 1 h (at most, overnight) and then centrifuged for 10 min to pellet the DNA. The DNA pellet was then resuspended in 400 µl of TE and 30 µg of RNase A was added and allowed

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to incubate at 37° C for 2 h. The sample was then extracted with 400 µl of chloroform:isoamyl alcohol (24:1). Following centrifugation, 350 µl of the aqueous layer was removed and placed into a new tube containing 1 mL of cold 100% ethanol and allowed to precipitate for at least 1 h at 20° C. After this second precipitation the DNA was pelleted and washed twice with 70% ethanol. The DNA pellet was air-dried at room temperature for 10 m and then resuspended in ultra pure water (80-100 µl). The quality and quantity of all samples were checked by gel electrophoresis.

Whole-genome DNA sequencing of yeast cells. For NGS, high-molecular weight genomic DNA (gDNA) was obtained from *pdr1Δ* benomyl and rapamycin resistant samples as described above. For the DNA library prep, 500 ng of gDNA was first sheared down to 300-400 bp using the Covaris S2 (Woburn, Massachusetts) according to the manufacture's recommendations. A target insert size of 300-400bp was then size-selected using an automated electrophoretic DNA fractionation system, LabChip XT (Caliper Life Sciences, Hopkinton, Massachusetts). Paired-end sequencing libraries were prepared using Illumina's TruSeq DNA Sample Preparation Kit (San Diego, CA). Following DNA library construction, samples were quantified using the Agilent Bioanalyzer per manufacturer's protocol (Santa Clara, CA). DNA libraries were sequenced using the Illumina HiSeq 2000 in one flow cell lane with sequencing paired-end read length at 2 x 100 bp. Reads were de-multiplexed using CASAVA (version 1.8.2).

Sequencing data analysis. Using the software tool Bowtie 2[27], we mapped the raw Illumina sequence data (as .fastq files representing all paired-end reads) from the drug-resistant mutants, as well as the parental strain *pdr1Δ*, to the most current *S. cerivisiae* reference genome assembly (sacCer3; April 2011). Sequencing was performed at a depth of 116 and 100 for the paired reads, and these were trimmed to 70 bases each for the mapping. We kept only the uniquely mapping reads to generate .bam files for each sample, including the parental strain. We found that 95% of the genome was covered by at least one read. These reads were then filtered to include only those that were inside the 1%-tile and 96%-tile in the read-depth distribution (see Supporting Information S1). We applied the

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genome analysis toolkit GATK[28, 29] to the .bam files from each mapped sample to produce SNP calls relative to the *sacCer3* reference genome. In order to generate SNP calls, the mapped files were processed using the GATK software to generate VCF files, using the following quality filters for calling SNPs: MQ < 30; FS > 60; ReadPosRankSum < -8.0. For each drug-resistant sample, we subtracted those SNPs that were also found in the *pdr1Δ* parental sample.

Results

Use of *pdr1* deletion strain as parental strain for mutant selection. In an earlier study, we had identified a number of novel cytotoxic compounds in *S. cerevisiae*[30] and had set out to identify their targets by selecting and sequencing drug-resistant mutants. In the first of these studies, we selected eight spontaneous mutants that were resistant to the drug of interest but remained sensitive to a panel of unrelated antifungal compounds. We anticipated that screening for cross-resistance against a diverse panel of unrelated drugs would allow us to eliminate any mutants that acquired resistance through multi-drug resistance (MDR) mechanisms, e.g., through up-regulation of drug efflux pumps or xenobiotic metabolism. Eight of the most promising drug-resistant mutants were selected based on a) resistance to the drug of interest and b) lack of resistance to the cross-screening panel. Sequencing the eight mutants plus the parental strain using NGS (SOLID) technology showed that, despite our efforts to eliminate MDR mutants, all eight of the resistant strains carried a mutation in the multidrug resistance gene *PDR1*. The *PDR1* gene encodes a transcription factor that regulates the expression of multi-drug resistance genes including drug efflux pumps in the *PDR* family. A variety of point mutations in *PDR1* are known to confer the MDR phenotype[31] and are clustered in distinct regions within *PDR1.* All eight of the *pdr1* point mutations in our drug-resistant samples also clustered in these regions (data not shown).

Different point mutations in *PDR1* are known to confer unique patterns of drug resistance, possibly due to the effect of each point mutation on the expression of specific ABC transporters[32]. Thus, while

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the drug resistance in these mutants appeared to be specific to our drug of interest, this specificity was probably due to the particular efficiency with which the drug was effluxed compared to the other drugs in the cross-screening panel, and not to a mechanism of resistance related to the drug's specific molecular target. These observations prompted us to select for resistance mutations in a *pdr1Δ* genetic background, which would not only eliminate *pdr1* mutations as sources of drug resistance, but would also make the yeast more drug sensitive in general. This enhanced sensitivity would allow us to use less compound in the selection experiments and could help to minimize other off-target effects. We next set out to test these hypotheses using two drugs whose targets are well established in yeast, benomyl and rapamycin.

Selection and cross-screening of benomyl- and rapamycin- resistant mutants. Equal aliquots of EMS-treated and -untreated *pdr1Δ* cells were plated onto YPD/agar with an optimal lethal dose of either benomyl or rapamycin, determined based on preliminary growth experiments with the parental strain (See Methods). 65 benomyl resistant colonies were isolated from the EMS-treated cells, whereas no resistant colonies arose from the non-EMS-treated cells. All 65 EMS-derived benomyl resistant mutants formed substantial colonies when subjected to a second round of selection on benomyl media, while the parental control strain produced no colonies. Rapamycin selection yielded only 5 resistant colonies, one from EMS-treated cells and four from non-EMS-treated cells. In a second round of selection on rapamycin plates, all six mutants formed normal sized colonies while the parental formed none.

Using an automated yeast halo assay that we had developed previously[33], we screened the benomyl- and rapamycin-resistant mutants for multi-drug resistance in the presence of 14 antifungal drugs representing a variety of MOA classes (Figure 2). Each mutant was seeded in agar and poured into a 384-well-format "omni-tray", and DMSO stock solutions of benomyl, rapamycin, and the 14 drugs in the cross-resistance panel were pin-transferred to each mutant tray. Mutants were chosen for sequencing based on two criteria: 1) They showed no discernable halo for the drug of interest (benomyl or rapamycin); and 2) On average, they were as sensitive as the parental control to the 14-drug panel.

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Based on the above criteria, 9 of the 65 benomyl-resistant mutants and 5 of the 6 rapamycin-resistant mutants were pooled and genomic DNA from the pools were prepared for sequencing using Illumina HiSeq 2000 (San Diego, Ca).

Sequencing of benomyl- and rapamycin- resistant mutants. We sequenced the pooled benomyl- and rapamycin-resistant mutants using the Illumina HiSeq 2000, and the reads were mapped onto the most current *S. cerevisiae* reference genome (sacCer3). The pool of 9 benomyl- and 5 rapamycin-resistant mutants were sequenced to average read depths of 211 and 199, respectively. When we compared the *pdr1Δ* sequence with that of the reference genome, ~20% of the 128 SNPs in the putatively haploid *pdr1Δ* appeared heterozygous. The detection of non-uniform SNPs in a haploid organism is consistent with reports from other genome-wide studies in yeast[24], in which spurious diploidization and transient polysomy has been known to occur during or prior to selection[34, 35]. Indeed, all of the SNPs that appeared heterozygous in the parental strain were also found at a similar allele frequency in the drug-selected pools. For this reason, all SNPs that were called in the parental strain were discarded, including at loci that appeared to be diploid.

After subtracting SNPs that were inherited from the parental strain, we obtained 1401 SNPs unique to the benomyl pool, averaging to ~156 SNPs per strain. The vast majority (97%) of these mutations were G-to-A and C-to-T transitions and distributed roughly evenly among the chromosomes. The number and type of mutations were consistent with previous reports on the base change frequency and specificity observed in EMS-treated yeast[36]. The SNPs were further filtered to remove synonymous and non-coding mutations, yielding a final list of 700 exonic SNPs mapping to 639 unique genes (Figure 3).

For each SNP, an "allele frequency" (AF) was calculated as the proportion of total reads at that locus carrying the alternate allele. Since each mutant contributed roughly the same amount of DNA to the pool, the number of strains carrying a particular SNP within the pool, i.e., the "allele count" (AC), could be estimated using the GATK software package. We initially set out to determine the significance of obtaining a particular AC by estimating probabilities based on the known mutation frequency and

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effective EMS-sensitive genome, assuming a random distribution of SNPs among the 9 strains (see Supporting Information, *statistical analysis*). If all the SNPs in the pool were distributed randomly over the 9 genomes in the pool, we calculate that observing even one mutation shared by two or more strains in the pool would occur in about 1 in 5 experiments. Finding three or more strains with the same mutation would occur by chance in only 2 in 10^5 experiments. And yet we observed 127 SNPs with AC values of 2, and 20 SNPs with AC values of 3. Many of these mutations were synonymous or occurred in non-coding regions, suggesting that they were not selective, and were most likely due to variations in the amount of DNA introduced per strain or variations among strains during the amplification of the DNA. Nonetheless, of the 700 SNPs in the benomyl-resistant pool, the SNP with the highest AC (AC = 4) was a C-to-T transition located in *TUB2*, the gene that encodes benomyl's known target, β-tubulin.

Multiple amino acid changes can confer drug resistance within the same target. Therefore, extending the allele count analysis to the gene level can, in principle, add another layer of confidence to the analysis by sidestepping the noise intrinsic to the calculated allele frequencies. For each of the 639 SNP-bearing genes from the benomyl pool, we created a new metric called the gene-level allele count (GL-AC), which represents the sum of the allele counts of all SNPs within a gene (see supplemental, information, *statistical analysis,* for formula). This gives us an upper bound on the number of strains with a mutation in a particular gene. The known benomyl target *TUB2* ranks highest among all genes with a GL-AC score of 8, which means that as many as 8 of the 9 strains might have mutations in that gene. The next-highest ranked genes were two genes with GL-AC scores of 5 (Table 1).

In the histogram of GL-AC scores presented in Figure 4, *TUB2* stands out among the other genes with its GL-AC score of 8. To determine if the GL-AC scores we observe are evidence of selection, we estimated the probability of observing any genes with GL-AC scores as large or larger than the ones we observe under non-selective conditions (i.e., the GL-AC distribution that one would expect if the 9 strains had been selected at random from a pool of EMS-treated strains under nonselective conditions). We did this by simulating the distribution of GL-ACs under the assumption that the SNPs are distributed randomly over the genes, taking into account gene length and base pair composition (based on the specificity of EMS-treatment for G and C (Supporting Information, *statistical analysis*)). This simulation

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showed that without selective pressure, from a pool of 9 EMS-treated strains finding *even one gene* with a GL-AC of 8 or higher would occur in only 8 out of 1000 experiments. On the other hand, finding at least a single gene with a GL-AC of 5 or higher would occur in 650 out of 1000 experiments. Therefore, *TUB2* is the only gene with a GL-AC value that is highly unlikely to have occurred by chance.

For the 5 pooled rapamycin mutants, one was derived from an EMS-treated line and the other four were spontaneous mutants. The genomic DNA of the 5 mutants were pooled in equal amounts and sequenced in the same manner as described above, to an average read depth of 199. SNP calls were performed using the same parameters as for the benomyl pool, yielding 116 SNPs that were not inherited from the parent. After filtering out synonymous and non-coding SNPs, 50 exonic, non-synonymous SNPs remained (Figure 5), each mapping to a unique gene. All but one of these SNPs had allele frequencies near 1/5 as depicted in the histogram in figure 6. The only SNP with an AC > 1 was located in the gene *FPR1* (AC=3), which encodes the yeast homolog of the human FK506-binding protein FKBP12, a well-known target of rapamycin (Table 2). Furthermore, there were two unique, non-parental alleles found *at the same locus* (an A-to-G and an A-to-T transition at position chrXIV:372100) proving that, at the very least, there were two strains harboring FPR1 SNPs. The probability of observing three or more strains with a mutation at the same base by chance is very low (*p*-value = 1.4×10^{-9}) given the low level of mutations in the genome and the fact that there are only 5 strains in the pool (Table 2). The two SNPs represent different but similar amino acid changes: Phe^{43}-to-Ile and Phe^{43}-to-Leu, located near the rapamycin binding pocket in the crystal structure (PDB 1FKB)[38]. Fpr1p binds rapamycin with high affinity, forming a toxic complex that binds and inhibits target of rapamycin proteins Tor1 and Tor2. Indeed, *TOR1* was among the 50 genes in the rapamycin-resistant pool that carried a mutation.

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Discussion

This proof-of-concept study provides a demonstration of the use of MUTseq for identifying drug targets in yeast. Analysis of the sequencing data from pools of benomyl- and rapamycin-resistant mutants resulted in a ranked list of genes for each drug, at the top of which were their known targets, *TUB2* and *FPR1*. Using MUTseq to confirm the target(s) of benomyl revealed three genes with GL-AC counts of five or more, with *TUB2*, the gene that encodes benomyl's known target, β-tubulin, at the top of the list. Interestingly, the most frequent alternate allele in *TUB2* that we identified corresponds to an Arg-to-Cys mutation at position 241 in β-tubulin, which is at the same site as a mutation (Arg to His) previously found in a screen for benomyl resistance[37].

Applying MUTseq to the antifungal, rapamycin revealed the gene *FPR1* (GL-AC=2), which encodes the homolog of the rapamycin- and FK506 binding protein FKBP12. Since four of the five strains in the rapamycin-resistant pool were selected from a set of spontaneous mutants, there were considerably fewer SNPs than in the benomyl pool. While the mutations that we identified in *FPR1* (F43I/L) has not been reported previously, in the crystal structure of the complex with FKBP12 (PDB 1FKB)[38], Phe^{43} projects directly into the FK506/rapamycin binding pocket. The SNP that we found in *TOR1* corresponds to the same mutation at Ser^{1972} that had been shown previously to confer resistance to rapamycin in yeast[16].

In the absence of selection, the likelihood of finding any SNP with an AC of greater than 2 in non-exonic bases in a pool of 9 strains is very low. It is unlikely that two or more of the benomyl mutants are clones since the EMS protocol used to introduce mutations does not allow for a recovery time after EMS treatment, precluding the cells from replicating and producing clones. A more likely explanation for unexpectedly large number of alternate alleles with AC = 2 and AC = 3 is that during pooling and library preparation prior to sequencing, one of the mutants had become disproportionately represented in the pool (e.g., from differential PCR amplification). Such differences in the relative contributions of strains within the pool, however, would have less of an impact on the interpretation of SNPs with higher AC values, especially ones that are rare. The only SNP with an AC = 4 in the benomyl pool was the R241C mutation in *TUB2*. If any such high-AC SNPs had arisen from an overrepresented strain, we

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would have expected other SNPs from this strain with similarly high AC values. GL-AC metric helps to mitigate against this source of error by providing an independent, gene-level analysis of SNPs based on the fact that multiple amino acid changes in the same protein can confer resistance to a drug.

In our selection of resistant mutants we found that the optimal selection conditions, as well as the mutation rates under each condition, were different for the two drugs tested. We obtained no spontaneous benomyl-resistant mutants, but isolated many EMS-derived mutants. In contrast, the majority of the rapamycin-resistant mutants that we identified were spontaneous, and the mutation rate for rapamycin was about 10-fold less than that of benomyl. This suggests that a variety of mutagenesis methods should be employed for each new drug to increase the likelihood of finding a constellation of resistance alleles for each drug. For example, UV-irradiation and proofreading-deficient *pol*δ mutants show different mutation specificities that are both somewhat orthogonal to that of EMS. Pools of resistant mutants derived from a variety of mutagens would increase the effective genome size available to absorb neutral mutations, while increasing the significance of any genes identified with high GL-AC values.

Of course the use of MUTseq requires that the compound of interest is lethal toward *S. cerevisiae*. While a given drug of interest may not be lethal toward wt yeast, or even toward classic MDR mutants like *pdr1Δ*, it may be possible to identify a yeast deletion mutant that is sensitive to the drug. An initial genome-wide search for sensitive haploid deletion mutants could be performed for a given compound using available techniques; such deletion mutants would provide the necessary genetic background for mutant selection, and in addition, the genome-wide sensitivity data could be useful in downstream MOA studies. In order to mitigate against identifying MDR resistance mutations in such cases, it would be advisable to knock out the gene that confers specific resistance in a *pdr1Δ* background.

Our results show that the sequencing of resistant strains of *S. cerevisiae* using NGS shows promise as a general method for identifying small molecule targets in this organism. The unbiased approach of prioritizing mutations, and the known targets that were uncovered in doing so, shows that this MUTseq can be applied to the discovery of new targets of novel compounds. In addition we show

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that by using the *pdr1Δ* background strain and screening for multi-drug resistance, we can minimize the occurrence of confounding MDR mutations.

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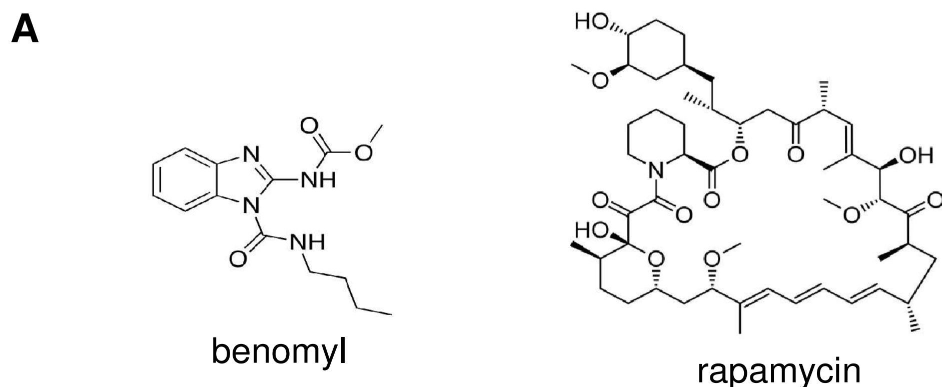
A



B

compound	Target gene	resistance mutation	Ref.
benomyl	*TUB2*	R241H	37
rapamycin	*TOR1*	S1972	16
	TOR2	S1975R	39
	FPR1	R49, F94	40

Figure 1. Benomyl and rapamycin are well-studied antifungals
(A) Structures of benomyl and rapamycin.
(B) A list of the genes that are known to encode the proteins that are targeted by benomyl and rapamycin. Also listed are residues that when modulated have been shown previously to confer resistance or inhibit drug binding.

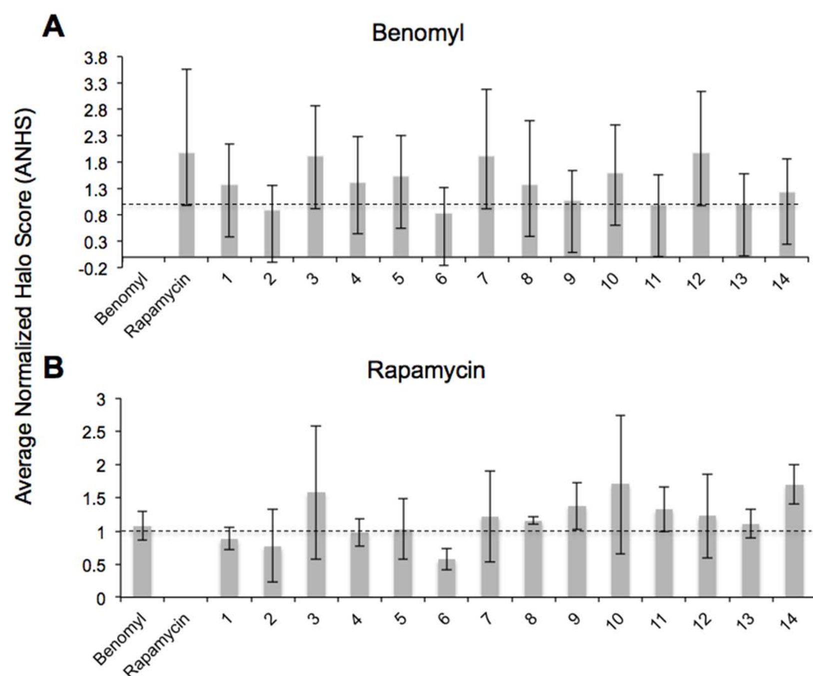


Figure 2. MDR cross resistance screen
(A) Chart displaying the average normalized halo score (ANHS) of the nine benomyl resistant mutants for 16 antifungals, including benomyl (first entry). ANHS values below one (dotted line) indicate resistance, and those above the line indicate sensitivity. Benomyl ANHS values of zero corresponds to an IC_{50} > 2mM.
(B) Chart displaying the ANHS of the five rapamycin resistant mutants for 16 antifugals including rapamycin (second entry). Rapamycin ANHS value of zero corresponds to an IC_{50} value of > 1 μM.

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A

	A	C	G	T
A		1	12	5
C	8		2	799
G	561	0		5
T	1	6	1	

Figure 3. Benomyl pool SNP statistics
(A) Matrix showing the base changes for all new SNPs (N=1401) in the benomyl pool.
(B) Mutations by region for all new SNPs in the benomyl pool.
(C) Distribution of exonic mutation types (N=1006).

B

Mutations by Region	Count
Exon	1006
Intron	12
Intergenic modifier	383

C

Exonic mutations	Count
missense	686
nonsense	14
synonymous	306

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Table 1: **Sequencing results for benomyl pool.** Highest ranking genes based on (gene level allele count) GL-AC.

gene	position	alternate reads/ total reads	AC	p-value (AC)	GL-AC	p-value (GL-AC)	mutation
TUB2	chrVI:57056	105/223	4	1.20E-09	8	0.008	**R241C**
	chrVI:57371	69/250	3	1.80E-05			P346S
	chrVI:56403	17/214	1	1			T23S
SCJ1	chrXIII:695582	38/125	3	1.80E-05	5	0.65	P78L
	chrXIII:696377	18/186	1	1			G343D
	chrXIII:6964	22/180	1	1			V359I
SPE4	chrXII:433410	58/195	3	1.80E-05	5	0.65	V106I
	chrXII:433661	51/209	2	0.17			S22N

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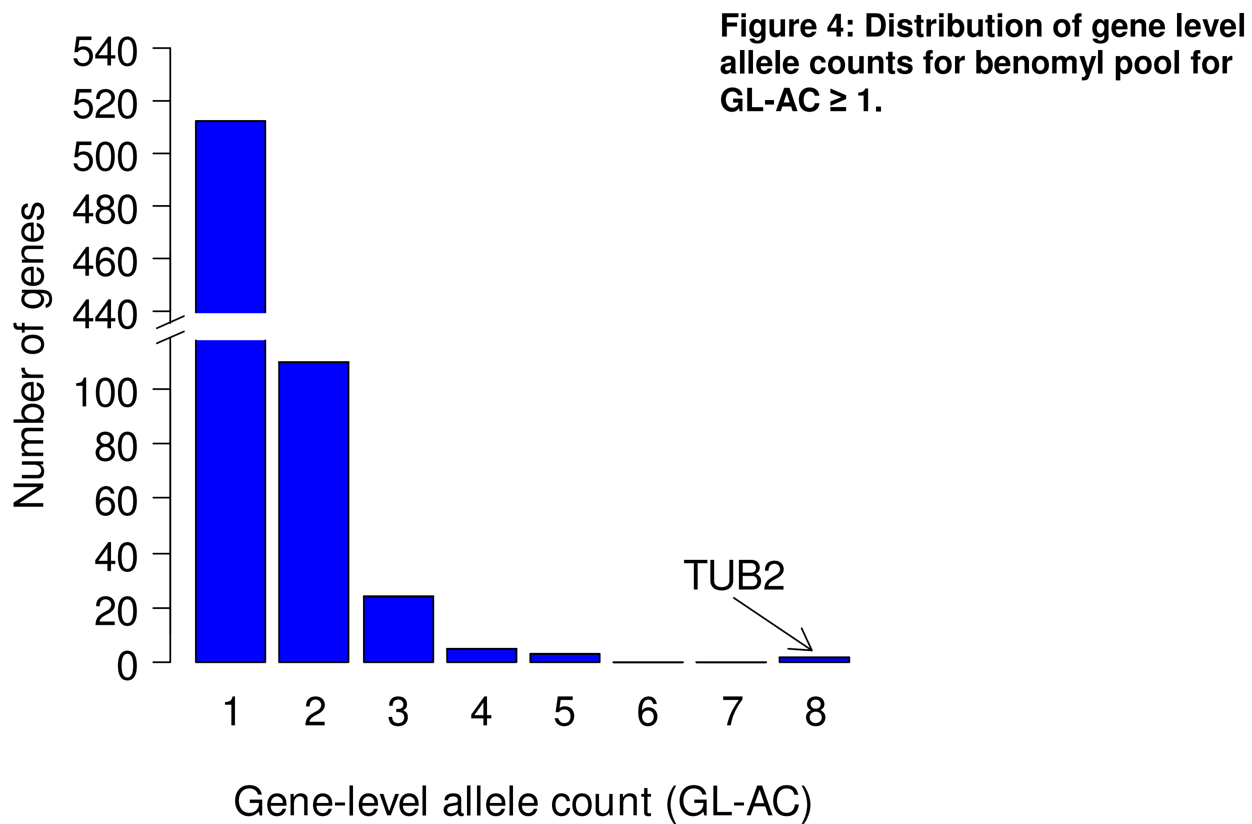

Figure 4: Distribution of gene level allele counts for benomyl pool for GL-AC ≥ 1.

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A

	A	C	G	G, T	T
A		1	4	1	2
C	6		1	0	44
G	35	0			8
T	1	2	1		

Figure 5. Rapamycin pool SNP statistics
(A) Matrix showing the base changes for all new SNPs (N=106) in the benomyl pool.
(B) Mutations by region for all new SNPs in the rapamycin pool.
(C) Distribution of exonic mutation types (N=64)

B

Mutations by Region	Count
Exon	64
Intron	0
Intergenic modifier	42

C

Exonic mutations	Count
missense	46
nonsense	4
synonymous	14

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Table 2: **Sequencing results for rapamycin pool.** Highest ranking genes based on (gene level allele count) GL-AC.

	gene	position	alternate reads/ total reads	AC	p-value (AC)	GL-AC	p-value (GL-AC)	mutation
1	**FPR1**	chrXIV:372100	38/63	3	1.40E-09	3	0.002	**F43I, F43L**
2	MZM	chrIV:1436553	38/177	1	1	1	1	A113S
3	DCR2	chrXII:847561	33/156	1	1	1	1	G522R
4	ITC1	chrVII:258208	44/212	1	1	1	1	R168C
5	ARO2	chrVII:226630	26/136	1	1	1	1	P78S
:	:							
:	:							
:	:							
16	TOR1	chrX:565331	33/204	1	1	1	1	S1972R
:	:							
:	:							
:	:							
50	NTE1	chrXIII:157425	12/221	1	1	1	1	D278E

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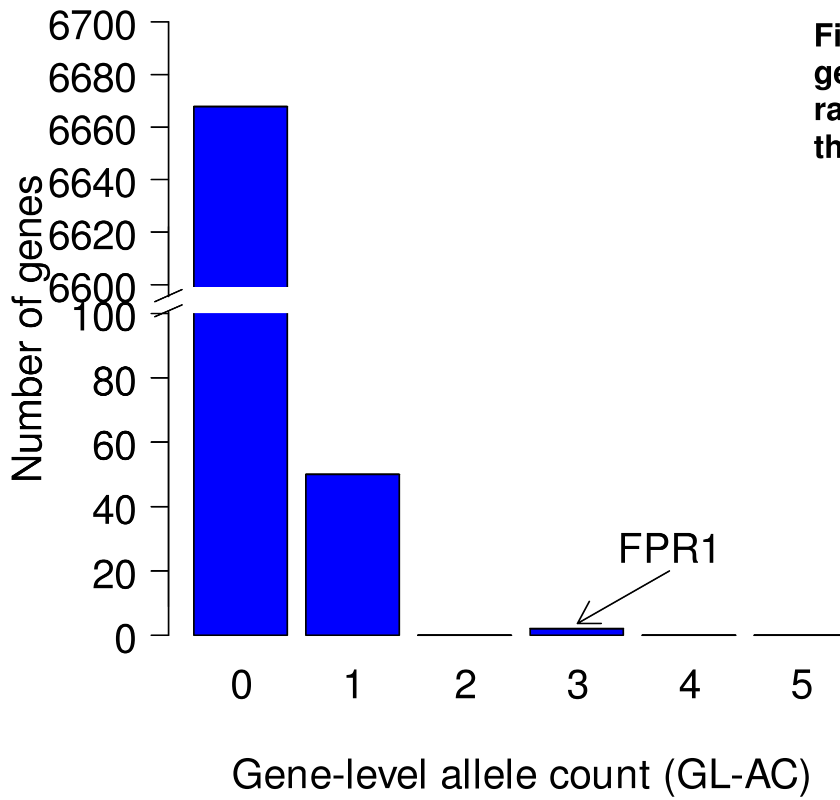

Figure 6: Distribution of gene level allele counts for rapamycin pool, including those with GL-AC = 0.

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