Supporting Materials

Figure S1. An alignment of 20 members of the Pdr5 subfamily of fungal ABC transporters. The atypical ATP-binding site and surrounding nucleotides including Gln-244 are composed of the Walker A, Walker B, and Q-loop regions of the N-terminal NBD and the signature region of the other NBD. These sequences are shown for this subfamily in the top-half of the alignment. The equivalent sequences for the canonical site including Glu-951 are shown in the bottom half.

Suppressors of N242K cyh hypersensitivity also fall in TMD1 and the deviant ATP-binding site. The N242K mutation confers significant cyh hypersensitivity *(6).* We isolated and mapped seven independent suppressors of this mutation in the *PDR5* gene. DNA sequencing established that all had the original N242K mutation as well as a second alteration. As shown in Figure S2A, all restore cyh resistance to a level that exceeds N242K and often even the WT (JG2015). A comparison of a chromosomal and the same mutation reconstructed in pSS607 (K1016I, N242K) using site-directed mutagenesis is also shown in Figure 2B. The location of these suppressors is found in Table S1. Aside from L806F which lies in the extracellular loop connecting TMD1 and 2, all of the suppressors are clearly located in the same half of Pdr5 as N242K. M649I and A666G are in TMH4 and 5 respectively, while V656L is in ICL2. The K1016I mutation is in NBD2, but it lies in the conserved signature residue toward the end of the region (in the Pdr5 family, both signature motifs have a conserved RKR triplet). In ABC transporters, the signature region of NBD2 forms a hybrid with the Walker A and B regions of NBD1 to constitute an ATP-binding site that would be cis with regard to Gln-242 and Ser-558.

Figure S2 . Suppressors of N242K cyh hypersensitivity. Ten cultures of N242K were started with 10^4 cells and grown to a concentration of ~1.5 X 10^7 cells/ml. 10^7 cells were plated from each culture on separate YPD plates containing 10 μM cyh. Plates were incubated for 96 hr at 30° C. A single colony was picked from each plate for further analysis. Genetic mapping established that seven of 10 mutants were due to an alteration of Pdr5. *A,* A quantitative analysis of all the original suppressors in liquid culture containing cyh as described in Figure 5B. *B,* The N242K, K1016I mutation was constructed in pSS607, placed in R-1 and compared to the chromosomal suppressor by evaluating the resistance of the strains to cyh. In all of the experiments, n = 3.

Figure S3. Qualitative analysis of chloramphenicol resistance in single and double Q-loop mutants. Serial-dilution spot test was carried out as described in the "Experimental Procedures". As chloramphenicol inhibits mitochondrial protein synthesis, the inhibitor (1.5 and 3.0 mM) was added to YPG medium and the plates

were scanned after 72 hr growth at 30° C. The experiment was repeated once with identical results. Strains are as follows: #1 = WT, #2 = S558Y , #3 = E244G, #4 = Q951G, #5 = E244G, Q951G

TABLE S1: Location and number of suppressors of N242K[a]

Residue alteration	Predicted location	No. found
M649I	TMH4	2
V656L	ICL2	1
A666G	TMH5	2
L806F	ECL1	1
K1016I	NBD2 Signature	1

[a] Mutations were isolated as described in Figure S2. DNA for sequencing was recovered from 5-FOA derivatives using the Clone Saver card and purification reagents available from Thomas Scientific (Swedesboro, N. J.). To amplify the entire *PDR5* orf, we used these primers; Pdr5-right, 5'TCGCATTTTGAGCAGTTTTG3' and pdr5-left, 5'GCCTTCGAGCACAGGATAA3'

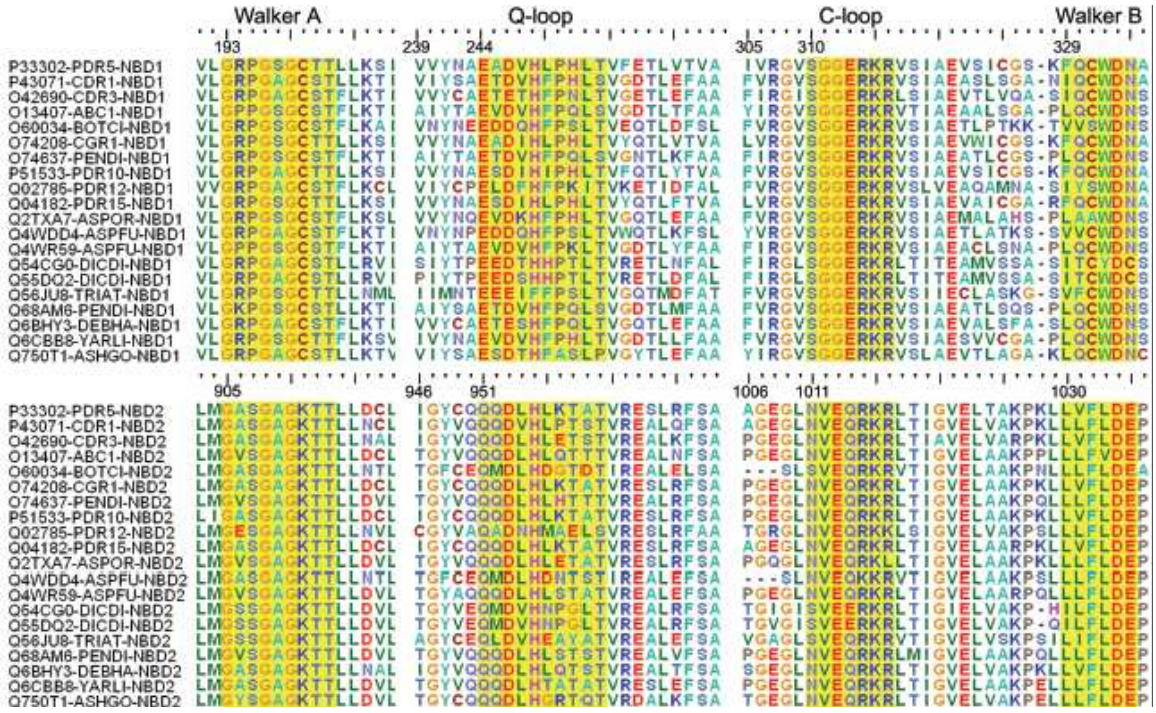
Walker A
Q-loop
C-loop
Walker B
193
239
244
305
310
329
P33302-PDR5-NBD1
P43071-CDR1-NBD1
Q42690-CDR3-NBD1
O13407-ABC1-NBD1
O60034-BOTCI-NBD1
O74208-CGR1-NBD1
O74637-PENDI-NBD1
P51533-PDR10-NBD1
Q02785-PDR12-NBD1
Q04182-PDR15-NBD1
Q2TXA7-ASPOR-NBD1
Q4WDD4-ASPFU-NBD1
Q4WR59-ASPFU-NBD1
Q54CG0-DICDI-NBD1
Q55DQ2-DICDI-NBD1
Q56JU8-TRIAT-NBD1
Q68AM6-PENDI-NBD1
Q6BHY3-DEBHA-NBD1
Q6CBB8-YARLI-NBD1
Q750T1-ASHGO-NBD1
905
946
951
1006
1011
1030
P33302-PDR5-NBD2
P43071-CDR1-NBD2
Q42690-CDR3-NBD2
O13407-ABC1-NBD2
O60034-BOTCI-NBD2
O74208-CGR1-NBD2
O74637-PENDI-NBD2
P51533-PDR10-NBD2
Q02785-PDR12-NBD2
Q04182-PDR15-NBD2
Q2TXA7-ASPOR-NBD2
Q4WDD4-ASPFU-NBD2
Q4WR59-ASPFU-NBD2
Q54CG0-DICDI-NBD2
Q55DQ2-DICDI-NBD2
Q56JU8-TRIAT-NBD2
Q68AM6-PENDI-NBD2
Q6BHY3-DEBHA-NBD2
Q6CBB8-YARLI-NBD2
Q750T1-ASHGO-NBD2

Fig. S1

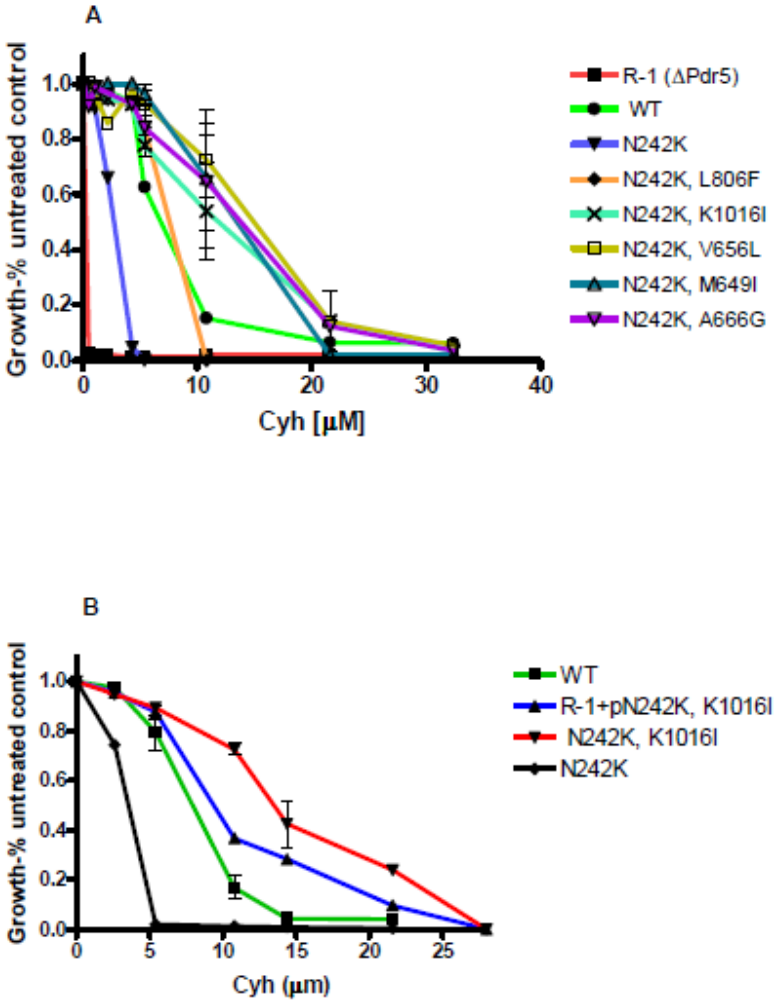
A
Growth-% untreated control
1.0
0.8
0.6
0.4
0.2
0.0
0
10
20
30
40
Cyh [µM]
R-1 (ΔPdr5)
WT
N242K
N242K, L806F
N242K, K1016I
N242K, V656L
N242K, M649I
N242K, A666G
B
Growth-% untreated control
1.0
0.8
0.6
0.4
0.2
0.0
0
5
10
15
20
25
Cyh (µm)
WT
R-1+pN242K, K1016I
N242K, K1016I
N242K

Fig. S2

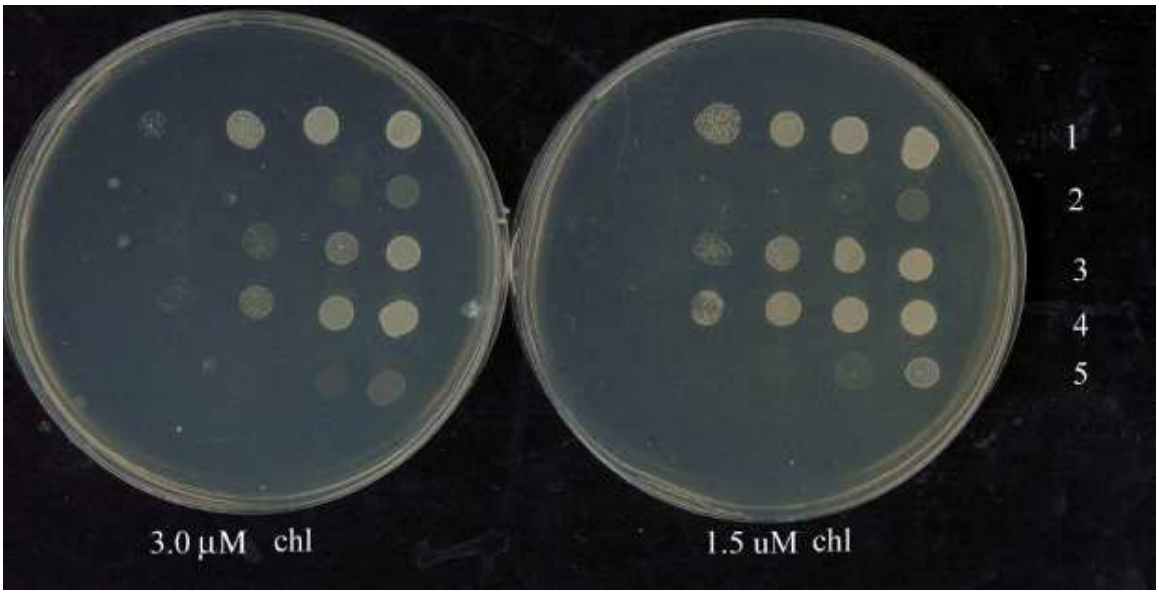

Fig. S3