Table S1. *S. cerevisiae* parental strains used in the study

Strain	Genotype	Source	Reference
ADΔ	MATα *PDR1-3 ΔYOR1::hisG ΔSNQ2::hisG ΔPDR3::hisG ΔPDR10::hisG ΔPDR11::hisG ΔYCF1::hisG ΔPDR55::hisG ΔPDR15::hisG Δura3 Δhis*AD124567 *ΔPDR5::hisG ΔPDR15::hisG, Δura3*	Lamping et al, 2007	[1]
Y941	ADΔ, Δpdr5::ScCYP51-6xHIS-URA3	Monk et al, 2014	[2]
Y1857	AD2Δ (ADΔ, ΔHIS1::dpl200)	Sagatova et al, 2016	[3]
Y2411	AD2Δ, ΔPDR5::URA3	E. Lamping	
Y2494	AD2Δ, ΔCYP51pro::GAL1pro	Monk et al, 2019	[4]

Table S2. Oligonucleotides used in the study

A. Construction of CpCYP51-6×His Y132F cassette by 3-fragment fusion for expression from the *PDR5* locus		
1.Amplification of CpCYP51-6×His Y132F ORF from plasmids received from ATUM		
Forward primer	PDR5us-CpCYP51 Y132F_f (52)	CCGCTCGTTCGAAAGACTTAATTAAAAAATGG CTTTGGTGGACTTGGCGTTG
Reverse primer	6 HisStop_r (26)	CGAATTTAATGATGATGGTGATGATG
2. Amplification of upstream fragments from gDNA of selected strain		
Forward primer	PDR5Fv3_f (23)	TCGCATTCTGCGCCTTCGAGCAC
Reverse primer	pABC3-PacI_r (31)	CATTTTTTAATTAAGTCTTTCGAACGAGCGG
3. Amplification of downstream fragment from gDNA of selected strain		
Forward primer	6HisStop-f (26)	CATCATCACCATCATCATTAAATTCG
Reverse primer	PDR5_186DS_r (25)	TTCGGACATTGAACTTTGATTTATC
B. For wild type strain construct reversion		
Forward primer	CpCyp51_Y132_f (31)	GTAAAGGTGTGATCTACGATTGTCCTAACGC
Reverse primer	CpCyp51_Y132_r (31)	GCGTTAGGACAATCGTAGATCACACCTTTAC
C. Construction of CpCPR-6×His cassette by 3-fragment fusion for expression from the *PDR15* locus		
1. Amplification of CpCPR-6×His ORF from plasmids received from ATUM		
Forward primer	PDR5us-CpCPR (54)	CCGCTCGTTCGAAAGACTTAATTAAAAAATGG CTTTAGATAGACTAGATCTTAC
Reverse primer	6 HisStop -r (26)	
2. Amplification of upstream fragments from gDNA of selected strain		
Forward primer	PDR15us_f (25)	GTCACGCCGCCGAACTGCAGCGCGC
Reverse primer	pABC3-PacI_r (31)	CATTTTTTAATTAAGTCTTTCGAACGAGCGG
3. Amplification of downstream fragment from gDNA of selected strain		
Forward primer	Not1-6xHis (34)	GGCGGCCGCCATCATCACCATCATCATTAAAT TC
Reverse primer	PDR15DS_r (25)	GATGGAATAATCCAGTTCGACTCTG
D. Amplification of ScHIS1 disruption cassette from gDNA of selected strains		

Forward primer	ScErg11_US-773_f (24)	GCAACAATGGGCGGTTGTTTAGAG
Reverse primer	ScErg11DS346_r (25)	GACTGCTTTATTTCtGCTTGGCCTG

*f and r denotes forward and reverse primer respectively. Number of nucleotides is shown in brackets.

Table S3. MIC_{80} values for strains overexpressing CpCYP51, CpCYP51 Y132F with/without CpCPR

Strains	MIC_{80}							
	FLC (μM)	**VCZ (nM)**	**ITC (nM)**	**PCZ (nM)**	**VT-1161 (nM)**	**VT-1129 (nM)**	**MCF (nM)**	**AmpB (μM)**
Y2411	2.4±0.08	41.7±1.6	128±12	157±3.4	27.1±0.5	26.7±0.2	212±15	2.69±0.2
Y2718	4.2±0.4	104±7.5	187±16	253±48	58±6.1	51.3±10	288±31	2.24±0.13
Y2719	4.65 ±0.13	131±3.6	222±16	188±9.6	58±8	41.6±3.5	263±18	2.74±0.08
Y2720	6.67 ±0.09	146±8.3	213±18	283±21	84.7±8	56.5±6.5	247±3.0	2.58±0.03
Y2721	4.97 ±0.03	121±2.03	291±3.3	229±3.5	46.8±2	47.5±8.8	237±1.5	2.77±0.05
Y2713	38.2±3.4	1280±81	202±1.4	164±4.7	199±5.4	264±24	237±3.2	2.8±0.02
Y2714	47.2±5.8	1820±18	207±3.6	242±17	277±8.1	367±16	280±1.5	2.09±0.2
Y2715	50±2.8	1540±44	201±0.7	179±0.5	222±20	299±21	184±16	3.38±0.3
Y2716	49±0.01	1410±20	202±0.13	228±25	295±3.8	293±5.2	205±0.9	2.9±0.2

MIC_{80}s are shown as the mean values ± SEM for 3 separate clones of each strain using data obtained in triplicate measurements from at least 3 different experiments (a total of 9 measurements per strain).

Table S4. Data collection and refinement statistics for ScCYP51-6×His in complex with VT-1129

Protein	ScCYP51-6×His
Ligand	VT-1129
PDB ID	7RYX
Data collection	
Diffraction source	MX2
Wavelength (Å)	0.9537
Space group	P 1 2_1 1
Cell dimensions	
a, b, c (Å)	78.4, 67.98, 80.3
α, β, γ (°)	90, 99.38, 90
Total reflections	279113
Unique reflections	48767
Resolution (Å)	2.1 - 45.19 (2.16- 2.10)
R_{merge}	0.086 (0.720)
I / σ I	8.6 (1.7)
Completeness (%)	99.8 (99.9)
Redundancy	5.7 (6.0)
$CC_{1/2}$	0.996 (0.893)
Refinement	
Resolution (Å)	2.1
No. reflections	48681
Rwork / Rfree	0.2336/0.2684
No. of atoms	
Protein	4271
Ligand/ion	79
Water	41
B-factors (Å^2)	
Protein	61.69
Ligand/ion	49.68
Water	52.63
R.m.s. deviations	
Bond lengths (Å)	0.008
Bond angles (°)	0.94
Ramachandran favored (%)	95.44
Ramachandran allowed (%)	3.99
Ramachandran outliers (%)	0.57
Rotamer outliers (%)	0.65
Clashscore	5.79

A. Mass spectrometry result of CpCYP51-6×His

Digestion of CpCYP51-6×His with trypsin

MALVDLALHGYNYFMTLSTLQQFGLLVFAPFIYNIIWQLLYSLRKDRVPLVFYWIPWVGSAVSYGQDPYGFFEQCREKYGDLF
SFVMLGRVMTVYLGPKGHEFVFNAKLSDVSAEDAYQHLTTPVFGKGVIYDCPNARLMEQKKFAKTALTTDSFRRYVPLIRGEI
LDYFTKSKVFNMKKQKSGVVDVLQSQPEITIFTASRSLLGEAMRKRFDASFAQLYADLDKGFTPINFVFPHLPLPHYWKRDAA
QQKISETYMTEIARRRETGDIDENRDLIDSLLVNSTYKDGVKMTDQEIANLLIGVLMGGQHTSATTSAWFLLHLAEKPQLQDE
LYQEVLNALSGKGGNLDDLSYEDLQQMPLVNNTIKETLRLHMPLHSIFRKVVSPLVVPNTKYIVPRGHHVLVSPGYAHTNERF
YKDASDFNPHRWDESASTNDAGEVDYGFGKVSKGVSSSYLPFGGGRHRCIGEQFAYVQLGTILTTFVYNLKWKLANGKVPDVD
YTSMVTLPQHPAEIVWEKRDTCVIGGRHHHHHH

Protein sequence coverage is 67.8%

Digestion of CpCYP51-6×His with chymotrypsin

MALVDLALHGYNYFMTLSTLQQFGLLVFAPFIYNIIWQLLYSLRKDRVPLVFYWIPWVGSAVSYGQDPYGFFEQCREKYGDLF
SFVMLGRVMTVYLGPKGHEFVFNAKLSDVSAEDAYQHLTTPVFGKGVIYDCPNARLMEQKKFAKTALTTDSFRRYVPLIRGEI
LDYFTKSKVFNMKKQKSGVVDVLQSQPEITIFTASRSLLGEAMRKRFDASFAQLYADLDKGFTPINFVFPHLPLPHYWKRDAA
QQKISETYMTEIARRRETGDIDENRDLIDSLLVNSTYKDGVKMTDQEIANLLIGVLMGGQHTSATTSAWFLLHLAEKPQLQDE
LYQEVLNALSGKGGNLDDLSYEDLQQMPLVNNTIKETLRLHMPLHSIFRKVVSPLVVPNTKYIVPRGHHVLVSPGYAHTNERF
YKDASDFNPHRWDESASTNDAGEVDYGFGKVSKGVSSSYLPFGGGRHRCIGEQFAYVQLGTILTTFVYNLKWKLANGKVPDVD
YTSMVTLPQHPAEIVWEKRDTCVIGGRHHHHHH

Protein sequence coverage is 88.32%

B. Mass spectrometry result of CpCYP51-6×His Y132F

Digestion of CpCYP51-6×His Y132F with trypsin. (The 'F' substitution at the 132 position is highlighted in red).

MALVDLALHGYNYFMTLSTLQQFGLLVFAPFIYNIIWQLLYSLRKDRVPLVFYWIPWVGSAVSYGQDPYGFFEQCREKYGDLF
SFVMLGRVMTVYLGPKGHEFVFNAKLSDVSAEDAYQHLTTPVFGKGVIFDCPNARLMEQKKFAKTALTTDSFRRYVPLIRGEI
LDYFTKSKVFNMKKQKSGVVDVLQSQPEITIFTASRSLLGEAMRKRFDASFAQLYADLDKGFTPINFVFPHLPLPHYWKRDAA
QQKISETYMTEIARRRETGDIDENRDLIDSLLVNSTYKDGVKMTDQEIANLLIGVLMGGQHTSATTSAWFLLHLAEKPQLQDE
LYQEVLNALSGKGGNLDDLSYEDLQQMPLVNNTIKETLRLHMPLHSIFRKVVSPLVVPNTKYIVPRGHHVLVSPGYAHTNERF
YKDASDFNPHRWDESASTNDAGEVDYGFGKVSKGVSSSYLPFGGGRHRCIGEQFAYVQLGTILTTFVYNLKWKLANGKVPDVD
YTSMVTLPQHPAEIVWEKRDTCVIGGRHHHHHH

Protein sequence coverage is 63.09%

Digestion of CpCYP51-6×His Y132F with chymotrypsin. (The 'F' substitution at the 132 position is highlighted in red).

MALVDLALHGYNYFMTLSTLQQFGLLVFAPFIYNIIWQLLYSLRKDRVPLVFYWIPWVGSAVSYGQDPYGFFEQCREKYGDLF
SFVMLGRVMTVYLGPKGHEFVFNAKLSDVSAEDAYQHLTTPVFGKGVIFDCPNARLMEQKKFAKTALTTDSFRRYVPLIRGEI
LDYFTKSKVFNMKKQKSGVVDVLQSQPEITIFTASRSLLGEAMRKRFDASFAQLYADLDKGFTPINFVFPHLPLPHYWKRDAA
QQKISETYMTEIARRRETGDIDENRDLIDSLLVNSTYKDGVKMTDQEIANLLIGVLMGGQHTSATTSAWFLLHLAEKPQLQDE
LYQEVLNALSGKGGNLDDLSYEDLQQMPLVNNTIKETLRLHMPLHSIFRKVVSPLVVPNTKYIVPRGHHVLVSPGYAHTNERF
YKDASDFNPHRWDESASTNDAGEVDYGFGKVSKGVSSSYLPFGGGRHRCIGEQFAYVQLGTILTTFVYNLKWKLANGKVPDVD
YTSMVTLPQHPAEIVWEKRDTCVIGGRHHHHHH

Protein sequence coverage is 93.97%

C. Mass spectrometry result of CpCPR-6×His

Digestion of CpCPR-6×His with trypsin.

MALDRLDLTVVIVLAVAVAAYFIKSQYFSKPESSGFLNTDTAGGNSRDILATLTKNHKNTLLLFGSQTGTAEDYCNKMSRELS
ARFGLKTMVADFADYDWDNFGDIKEDVLVFFIMATYGEGEPTDNAIEFVDFLDNEADTLSTLRFTVFGLGNSTYEFFNAIGRK
INEKLESKGAERFAEYGEGDDGQGTMDEDFLAWKDGVFDSLRNNLNLEEKELKYEPSLKLEIRDDLTIDDSSVSLGEPDKSYV
NTKAGTDLTKGPFDHSHPYLAPITKIKELFFTKERSCVHVEFDLSNSNLKYTTGDHLAIWPSNANEYVELFLKTFDLTEQRDV
VFDLKALDSTYQIPFPTPITYEAVVRHHLEISGPVSRQFFLSIAAFAPDEETKTKLTTVANDKQKYAEEVTHKKYNIADGLLY
FSNGKPWTKVPFEFLIENVQHFTPRYYSISSSSLSEKTHIDITAVVEAETESDGRVVTGVVTNLLKDVEINKNSSSDKPIVSY
DLKGPRNKFQNYKLPVHVRRSTFKLPSSSKTPIILVGPGTGVAPLRGFVRERVQQLKNGVNVGPSLLFYGCRNEDEDYLYRDE
WPQYAKELGESFELITAFSRANPNKKVYVQHKILEQAKKINQLLQDGGIIYVCGDASHMARDVQASFAKVLSQERGIELEKAA
ELIRSLKVQNRYQEDVWGGRHHHHHH

Protein sequence coverage is 90.14%.

Digestion of CpCPR-6×His with chymotrypsin.

MALDRLDLTVVIVLAVAVAAYFIKSQYFSKPESSGFLNTDTAGGNSRDILATLTKNHKNTLLLFGSQTGTAEDYCNKMSRELS
ARFGLKTMVADFADYDWDNFGDIKEDVLVFFIMATYGEGEPTDNAIEFVDFLDNEADTLSTLRFTVFGLGNSTYEFFNAIGRK
INEKLESKGAERFAEYGEGDDGQGTMDEDFLAWKDGVFDSLRNNLNLEEKELKYEPSLKLEIRDDLTIDDSSVSLGEPDKSYV
NTKAGTDLTKGPFDHSHPYLAPITKIKELFFTKERSCVHVEFDLSNSNLKYTTGDHLAIWPSNANEYVELFLKTFDLTEQRDV
VFDLKALDSTYQIPFPTPITYEAVVRHHLEISGPVSRQFFLSIAAFAPDEETKTKLTTVANDKQKYAEEVTHKKYNIADGLLY
FSNGKPWTKVPFEFLIENVQHFTPRYYSISSSSLSEKTHIDITAVVEAETESDGRVVTGVVTNLLKDVEINKNSSSDKPIVSY
DLKGPRNKFQNYKLPVHVRRSTFKLPSSSKTPIILVGPGTGVAPLRGFVRERVQQLKNGVNVGPSLLFYGCRNEDEDYLYRDE
WPQYAKELGESFELITAFSRANPNKKVYVQHKILEQAKKINQLLQDGGIIYVCGDASHMARDVQASFAKVLSQERGIELEKAA
ELIRSLKVQNRYQEDVWGGRHHHHHH

Protein sequence coverage is 94.49%

Figure S1 Identification of the recombinant proteins by mass spectrometry. Sequences identified on excision from SDS-PAGE gel bands are highlighted in grey.

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