

## Supplementary materials: Graham et al.

**Table S1. Yeast strains used in this study.**

| Strain                              | Genotype  | Reference                                     |
|-------------------------------------|---|---|
| <b>ADΔ</b>                          | MATα PDR1-3 Δyor1::hisG<br>Δsnq2::hisG<br>Δpdr3::hisG Δpdr10::hisG<br>Δpdr11::hisG<br>Δycf1::hisG Δpdr5::hisG Δpdr15::hisG<br>Δura3 ΔhisAD124567 Δpdr5::hisG<br>Δpdr15::hisG, Δura3::dpl200 | Lamping <i>et al.</i><br>(2007) <sup>1</sup>  |
| <b>AD2Δ</b>                         | ADΔ, Δhis1::dpl200  | Sagatova <i>et al.</i><br>(2015) <sup>2</sup> |
| <b>AD3Δ ScErg11</b>                 | AD2Δ, Δpdr5::ScERG11-URA3,<br>Δerg11::HIS1  | Sagatova <i>et al.</i><br>(2015) <sup>2</sup> |
| <b>AD2Δ ScErg11 Y140H</b>           | AD2Δ, Δpdr5::ScERG11_Y140H-<br>URA3   | This study                                    |
| <b>AD3Δ ScErg11 Y140H</b>           | AD2Δ Δpdr5::ScERG11_Y140H,<br>Δerg11::HIS1  | This study                                    |
| <b>AD2Δ ScErg11 I471T</b>           | AD2Δ, Δpdr5::ScERG11_I471T-<br>URA3   | This study                                    |
| <b>AD3Δ ScErg11 I471T</b>           | AD2Δ Δpdr5::ScERG11_I471T,<br>Δerg11::HIS1  | This study                                    |
| <b>AD2Δ ScErg11 Y140H<br/>I471T</b> | AD2Δ,<br>Δpdr5::ScERG11_Y140H+I471T-<br>URA3  | This study                                    |
| <b>AD3Δ ScErg11 Y140H<br/>I471T</b> | AD2Δ<br>Δpdr5::ScERG11_Y140H+I471T,<br>Δerg11::HIS1   | This study                                    |

**Table S2. Oligonucleotide primers used in the study**

### *Creation of LDM6×His I471T*

Forward primer ScErg11p\_I471T\_f  
GGTGGTGGTAGACACAGATGTACCGGTGAACACTTTGCTTAC

Reverse primer ScErg11p\_I471T\_r  
GTAAGCAAAGTGTTCCACCGGTACATCTGTGTCTACCACCACC

### *Creation of LDM6×His Y140H*

Forward primer ScErg11p\_Y140H\_f  
AAGGTGTTATTCATGATTGTCCAAATTC

Reverse primer ScErg11p\_Y140H\_r  
TTGGACAATCATGAATAACACCTTTACC

**Table S3. Data collection and refinement statistics for ScLDM6×His Y140H in complex with VCZ, ScLDM6×His I471T in complex with ITC and VCZ, and ScLDM6×His Y140H + I471T in complex with VCZ**

| Protein                               | Y140H VCZ                          | I471T VCZ                          | I471T ITC                          | Y140H+I471T VCZ                 |
|---------------------------------------|------------------------------------|------------------------------------|------------------------------------|---------------------------------|
| PDB ID                                | 7RY8                               | 7RY9                               | 7RYA                               | 7RYB                            |
| Space group                           | P 1 2 <sub>1</sub> 1               | P 1 2 <sub>1</sub> 1               | P 1 2 <sub>1</sub> 1               | P 1 2 <sub>1</sub> 1            |
| Resolution range (Å)                  | 39.99 – 1.98 (2.03 – 1.98)         | 33.15 – 2.4 (2.49 – 2.40)          | 33.42 – 2.1 (2.18 – 2.10)          | 33.75 - 2.9 (3.00 – 2.90)       |
| Unit cell axes (Å)                    | a = 77.79, b = 66.86, c =<br>80.88 | a = 76.79, b = 66.31, c =<br>80.73 | a = 77.53, b = 66.70, c =<br>80.58 | a = 79.18, b = 67.30, c = 81.33 |
| Total reflections                     | 293230                             | 62937                              | 90476                              | 36666                           |
| Unique reflections                    | 57296                              | 31547                              | 45698                              | 18602                           |
| Completeness (%)                      | 99.8 (99.90)                       | 99.64 (99.90)                      | 95.73 (85.33)                      | 98.21                           |
| <I/σ(I)>                              | 7.2 (0.98)                         | 13.19 (2.01)                       | 13.86 (2.39)                       | 9.00 (1.43)                     |
| Wilson B-factor                       | 43.10                              | 38.91                              | 27.94                              | 54.02                           |
| R <sub>merge</sub>                    | 0.147 (3.302)                      | 0.044 (0.380)                      | 0.037 (0.285)                      | 0.067 (0.566)                   |
| CC(1/2)                               | 0.996 (0.317)                      | 0.998 (0.719)                      | 0.998 (0.836)                      | 0.992 (0.753)                   |
| Refinement                            |                                    |                                    |                                    |                                 |
| R <sub>work</sub>                     | 0.209 (0.362)                      | 0.199 (0.282)                      | 0.197 (0.259)                      | 0.201 (0.294)                   |
| R <sub>free</sub>                     | 0.234 (0.357)                      | 0.237 (0.307)                      | 0.235 (0.308)                      | 0.252 (0.353)                   |
| Number of reflections                 | 57118 (5548)                       | 31538 (3119)                       | 45680 (4038)                       | 18592 (1830)                    |
| Number of atoms in model              |                                    |                                    |                                    |                                 |
| Protein                               | 4478                               | 4262                               | 4280                               | 4228                            |
| Ligand                                | 96                                 | 81                                 | 92                                 | 68                              |
| Water molecules                       | 123                                | 67                                 | 196                                | 1                               |
| Deviation from ideal bond lengths (Å) | 0.007                              | 0.008                              | 0.008                              | 0.009                           |
| Deviation from ideal bond angles (°)  | 0.84                               | 0.93                               | 0.90                               | 1.02                            |
| Ramachandran analysis (%)             |                                    |                                    |                                    |                                 |
| Preferred                             | 97.14                              | 95.62                              | 97.15                              | 92.90                           |
| Allowed                               | 2.67                               | 4.00                               | 2.66                               | 6.72                            |
| Residues in disallowed regions        | 0.19                               | 0.38                               | 0.19                               | 0.38                            |

Statistics for the highest resolution shell shown in parentheses. One crystal was used per structure.  $R_{\text{free}}$  was calculated using a test set composed of 5% of data.

This research was undertaken on the MX1 beamline at a wavelength of 0.954 Å at the Australian Synchrotron, part of ANSTO<sup>3</sup>.

# Confirmation of LDM mutations by mass spectrometry of tryptic or chymotryptic fingerprints

Mass spectrometry (MS-MS) analysis of tryptic or chymotryptic fragments of the 62 kDa protein band gave protein sequence coverage of at least 54% of the ScLDM6×His primary sequence and detected the peptides showing the expected Y140H and I471T mutations (Figure S1).

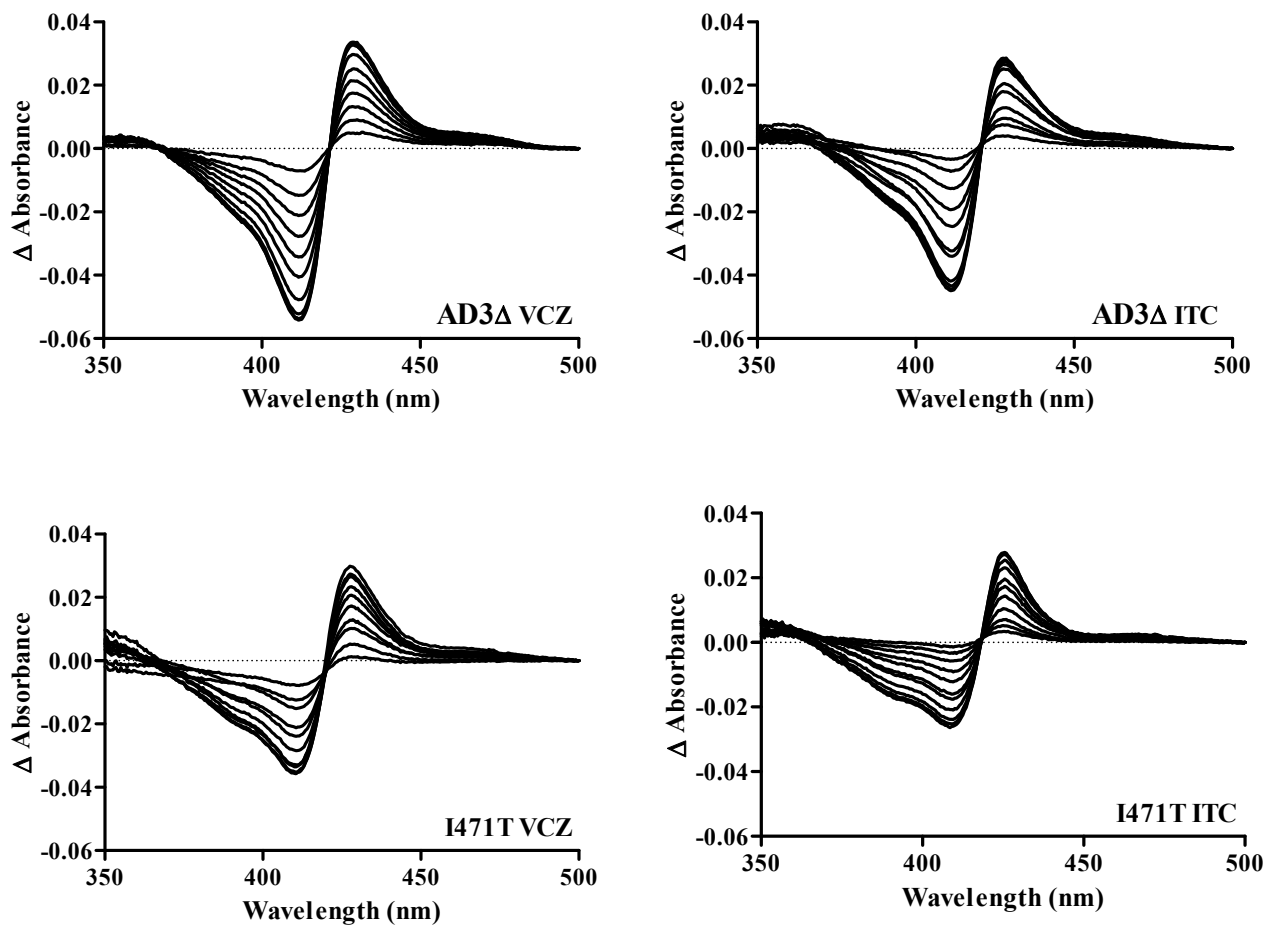
|           |             |             |             |             |
|-----------|-------------|-------------|-------------|-------------|
| <b>A.</b> |             |             |             |             |
| 11        | MSATKSIVGE  | ALEYVNIGLS  | HFLALPLAQR  | ISLIIIIIPFI |
| 441       | YNIVWQLLYS  | LRKDRPPLVF  | YWIPWVGS AV | VYGMKPYEFF  |
| 81        | EECQKKYGDI  | FSFVLLGRVM  | TVYLGPKGHE  | FVFNAKLADV  |
| 121       | SAEAAYAHLT  | TPVFGKGVIF  | DCPNSRLMEQ  | KKFVKGALTK  |
| 161       | EAFKSYVPLI  | AEEVYKYFRD  | SKNFRLNERT  | TGTIDVMVTQ  |
| 201       | PEMTIFTASR  | SLLGKEMRAK  | LDLDFAYLYS  | DLDKGFTPIN  |
| 241       | FVFPNLPLEH  | YRKRDHAQKA  | ISGTYMSLIK  | ERRKNNDIQD  |
| 281       | RDLIDSLMKN  | STYKDGVKMT  | DQEIANLLIG  | VLMGGQHTSA  |
| 321       | ATSAWILLHL  | AERP DVQQEL | YEEQMRVLDG  | GKKELTYDLL  |
| 361       | QEMPLLNQTI  | KETLRMHHPL  | HSLFRKVMKD  | MHVPNTSYVI  |
| 401       | PAGYHVLVSP  | GYTHLRDEYF  | PNAHQFNIHR  | WNND SASSYS |
| 441       | VGEEVDYGF G | AISKGVSSPY  | LPFGGGRHRC  | IGEHFAYCQL  |
| 481       | GVLMSIFIRT  | LKWHYPEGKT  | VPPPDFTSMV  | TLPTGPAKII  |
| 521       | WEKRNPEQKI  | GGRHHHHHH   |             |             |
| <b>B.</b> |             |             |             |             |
| 1         | MSATKSIVGE  | ALEYVNIGLS  | HFLALPLAQR  | ISLIIIIIPFI |
| 41        | YNIVWQLLYS  | LRKDRPPLVF  | YWIPWVGS AV | VYGMKPYEFF  |
| 81        | EECQKKYGDI  | FSFVLLGRVM  | TVYLGPKGHE  | FVFNAKLADV  |
| 121       | SAEAAYAHLT  | TPVFGKGVII  | DCPNSRLMEQ  | KKFVKGALTK  |
| 161       | EAFKSYVPLI  | AEEVYKYFRD  | SKNFRLNERT  | TGTIDVMVTQ  |
| 201       | PEMTIFTASR  | SLLGKEMRAK  | LDLDFAYLYS  | DLDKGFTPIN  |
| 241       | FVFPNLPLEH  | YRKRDHAQKA  | ISGTYMSLIK  | ERRKNNDIQD  |
| 281       | RDLIDSLMKN  | STYKDGVKMT  | DQEIANLLIG  | VLMGGQHTSA  |
| 321       | ATSAWILLHL  | AERP DVQQEL | YEEQMRVLDG  | GKKELTYDLL  |
| 361       | QEMPLLNQTI  | KETLRMHHPL  | HSLFRKVMKD  | MHVPNTSYVI  |
| 401       | PAGYHVLVSP  | GYTHLRDEYF  | PNAHQFNIHR  | WNND SASSYS |
| 441       | VGEEVDYGF G | AISKGVSSPY  | LPFGGGRHRC  | TGEHFAYCQL  |
| 481       | GVLMSIFIRT  | LKWHYPEGKT  | VPPPDFTSMV  | TLPTGPAKII  |
| 521       | WEKRNPEQKI  | GGRHHHHHH   |             |             |
| <b>C.</b> |             |             |             |             |
| 1         | MSATKSIVGE  | ALEYVNIGLS  | HFLALPLAQR  | ISLIIIIIPFI |
| 41        | YNIVWQLLYS  | LRKDRPPLVF  | YWIPWVGS AV | VYGMKPYEFF  |
| 81        | EECQKKYGDI  | FSFVLLGRVM  | TVYLGPKGHE  | FVFNAKLADV  |
| 121       | SAEAAYAHLT  | TPVFGKGVIF  | DCPNSRLMEQ  | KKFVKGALTK  |
| 161       | EAFKSYVPLI  | AEEVYKYFRD  | SKNFRLNERT  | TGTIDVMVTQ  |
| 201       | PEMTIFTASR  | SLLGKEMRAK  | LDLDFAYLYS  | DLDKGFTPIN  |
| 241       | FVFPNLPLEH  | YRKRDHAQKA  | ISGTYMSLIK  | ERRKNNDIQD  |
| 281       | RDLIDSLMKN  | STYKDGVKMT  | DQEIANLLIG  | VLMGGQHTSA  |
| 321       | ATSAWILLHL  | AERP DVQQEL | YEEQMRVLDG  | GKKELTYDLL  |
| 361       | QEMPLLNQTI  | KETLRMHHPL  | HSLFRKVMKD  | MHVPNTSYVI  |
| 401       | PAGYHVLVSP  | GYTHLRDEYF  | PNAHQFNIHR  | WNND SASSYS |
| 441       | VGEEVDYGF G | AISKGVSSPY  | LPFGGGRHRC  | TGEHFAYCQL  |
| 481       | GVLMSIFIRT  | LKWHYPEGKT  | VPPPDFTSMV  | TLPTGPAKII  |
| 521       | WEKRNPEQKI  | GGRHHHHHH   |             |             |

**Figure S1: Mass spectrometry analysis of proteolytic fingerprints of ScLDM6×His.** Tryptic (A and C) and chymotryptic (B) digests of the 62-kDa protein band excised from the SDS-PAGE gel profiles of crude membrane samples were analysed using Orbitrap-based mass spectrometry at the University of Otago, Department of Biochemistry Centre for Protein Research. Protein bands were digested using the DigestPro MSi (Intavis AG, Cologne, Germany) robotic workstation according to an established protocol <sup>4</sup>. The Mascot search engine was used to search the data obtained from the SWISS-PROT amino acid sequence database. The amino acid sequences of protein fragments from the ScLDM6×His mutants identified in the proteolytic digests are highlighted in bold. **(A)** The ScLDM6×His Y140H sample showed 54% coverage of the primary sequence. The Y140H mutation is highlighted in green. **(B)** The ScLDM6×His I471T sample showed 94.8% coverage of the primary sequence. The I471T mutation is highlighted in blue. **(C)** The ScLDM6×His Y140H + I471T sample showed 98.9% coverage. The Y140H and the I471T mutation are highlighted in green and blue, respectively.

*Assessment of in vitro binding affinity of test azoles to Ni-NTA affinity purified ScLDM6×His*

The affinity of the test azoles for Ni-NTA affinity purified ScLDM6×His was measured using an *in vitro* azole-binding assay (**Table S2**). We previously reported there were no significant differences between the  $K_d$  values (0.5  $\mu$ M range) for wild-type and Y140F/H mutants of ScLDM6×His binding to FLC, VCZ, ITC, and PCZ <sup>5</sup>. Because of lower stability of the detergent solubilised ScLDM6×His Y140H I471T, *in vitro* drug binding assays using the short-tailed azole VCZ and the long-tailed azole ITC were conducted for control wild-type ScLDM6×His (AD3Δ) and ScLDM6×His I471T preparations only (**Figure 4, figure S2 and Table S2**). Both ITC and VCZ showed tight type II binding to wild-type and I471T mutant ScLDM6×His, with  $[Azole]_{0.5}$  values of  $\sim 0.75$   $\mu$ M in the presence of 2  $\mu$ M functional enzyme and  $K_d$  values in the 0.5  $\mu$ M range. Other studies failed to find a correlation between whole cell drug susceptibility assays and azole affinity for purified LDM, including CaLDM containing the Y132H and I471T mutations <sup>5, 6</sup>. Sagatova *et al.* (2016) hypothesised that this may be due to the formation of near stoichiometric LDM-azole complexes that limit accuracy in the calculation of azole affinities <sup>5</sup>. It has also been shown that detergent solubilisation of *A. fumigatus* alters the ligand binding properties of the enzyme <sup>7</sup>. This is unsurprising as the X-ray crystal structure of full-length ScLDM6×His revealed rigid anchorage in the lipid bilayer of the mouth of the substrate entry channel that enables substrates and azole drugs to reach the

buried active site (PDB ID: 4LXJ) <sup>8, 9</sup>. Detergent solubilisation of ScLDM6×His could perturb this entry route to the active site and may explain the apparent lack of correlation between the results of whole cell drug susceptibility assays and *in vitro* azole binding assays. While the modest Hill numbers (1.55 – 1.85) obtained from the binding curves (**Figure 4**) might indicate cooperative binding between ScLDM6×His monomers, Sagatova *et al.* (2016) hypothesised that such values are more likely to represent multiple interactions between the azole and the internal active site of ScLDM6×His <sup>5</sup>



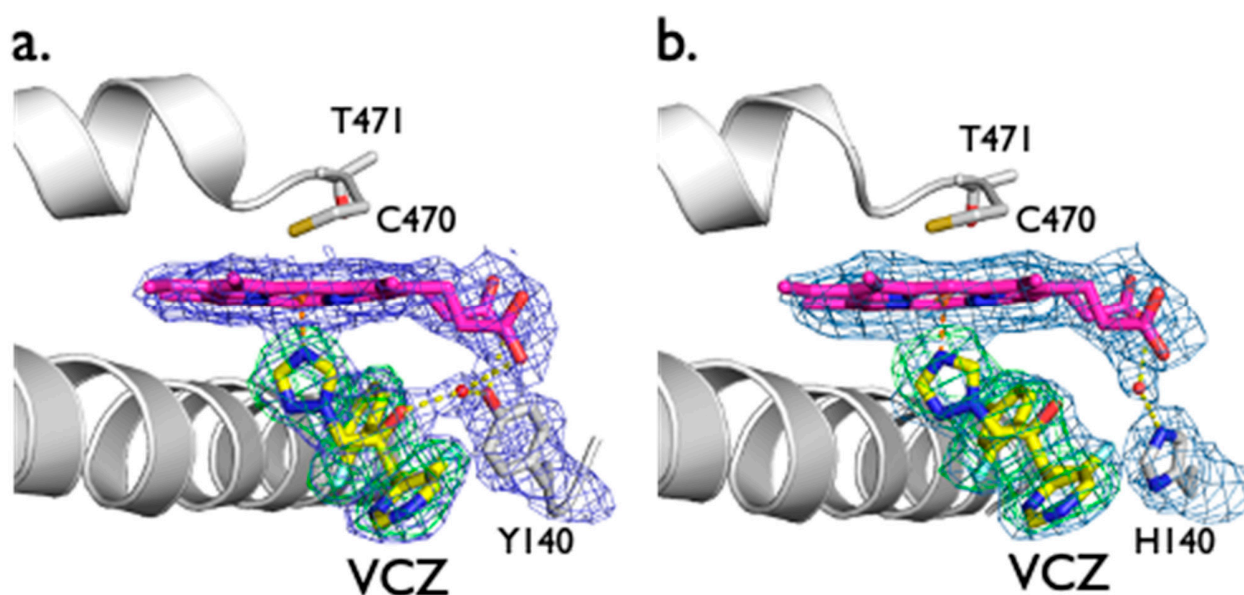
**Figure S2. Difference spectra showing type II binding of VCZ and ITC to wild-type (AD3 $\Delta$ ) ScErg11p6 $\times$ His and ScErg11p6 $\times$ His I471T mutant.** Difference spectra were obtained by titrating a 0.5 mL sample of 2  $\mu$ M functional ScErg11p6 $\times$ His as described in Materials and Methods.

**Table S4: Azole binding to affinity purified wild-type (AD3Δ), ScLDM6×His and ScLDM6×His I471T mutant.**

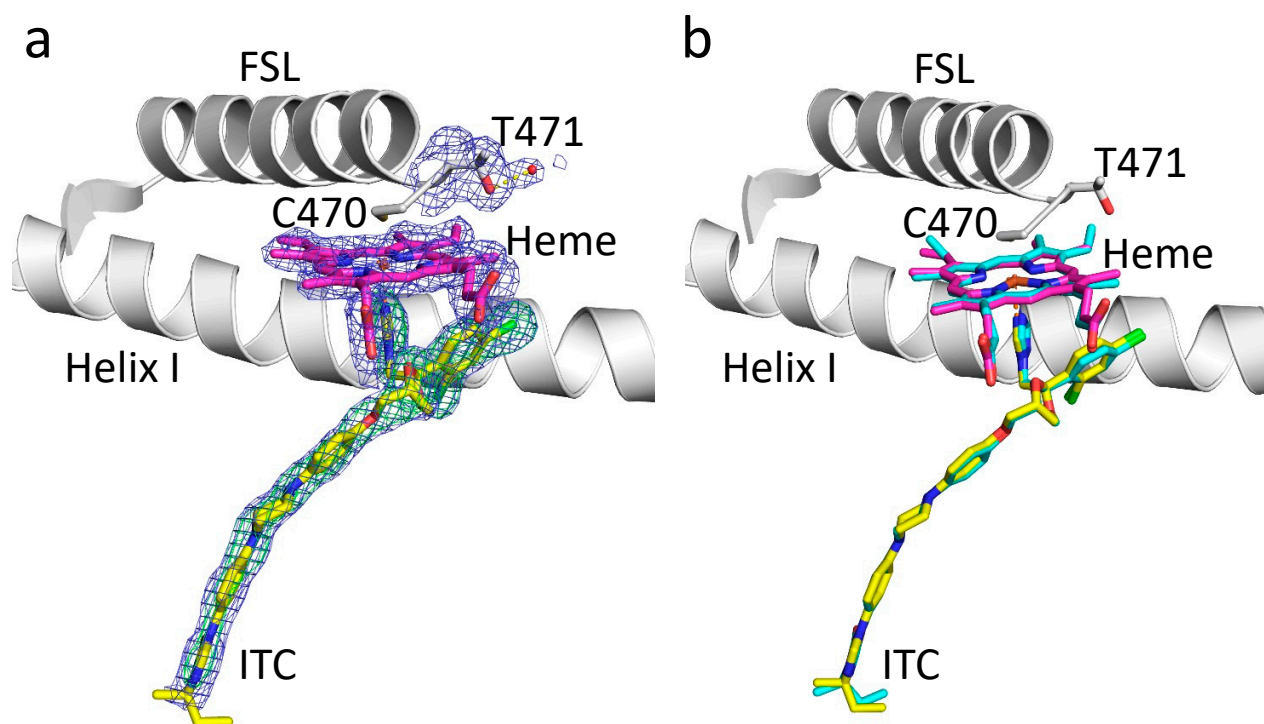
| ScLDM6×His | Azole | $\Delta A_{\max}$ | [Azole] <sub>0.5</sub> | $K_d$ (μM)  | Hill number |
|------------|-------|-------------------|------------------------|-------------|-------------|
| AD3Δ       | VCZ   | 0.10 (0.004)      | 0.78 (0.06)            | 0.62 (0.11) | 1.85 (0.23) |
|            | ITC   | 0.08 (0.006)      | 0.60 (0.07)            | 0.43 (0.12) | 1.61 (0.22) |
| I471T      | VCZ   | 0.07 (0.003)      | 0.75 (0.05)            | 0.58 (0.10) | 1.88 (0.23) |
|            | ITC   | 0.06 (0.001)      | 0.79 (0.10)            | 0.70 (0.16) | 1.55 (0.19) |

Values in brackets represent  $\pm$  standard errors of the mean.





**Figure S3. OMIT maps for the binding VCZ to (a) ScLDM6×His I471T and (b) ScLDM6×His Y140H + I471T.** VCZ is shown as sticks, C atoms yellow, N atoms blue, O atoms red, F atoms pale blue. The heme cofactor is shown with C atoms in magenta. Electron density is shown for VCZ, heme, water molecules, and the site of the native Y140 residue and H140 mutation. The  $2F_o - F_c$  electron density map (blue) is contoured at  $1\sigma$  and the  $F_o - F_c$  map (green) is contoured at  $3\sigma$ . OMIT maps were generated by Phenix and visualised in PyMOL. The  $F_o - F_c$  map was calculated using  $F_{calc}$  from the refinement process with no ligand in the active site. The  $2F_o - F_c$  map was calculated following final refinement after modelling VCZ in the active site. Water molecules are shown as red spheres. Hydrogen bonds are shown as dashed yellow lines.



**Figure S4. Binding of ITC to ScLDM6×His I471T.** (a) OMIT map is shown for ITC, the heme, and T471 with associated water molecule. ITC is shown as sticks, C atoms in yellow, N atoms in blue, O atoms in red, Cl atoms in green. The heme is shown with C atoms in magenta. The  $2F_o - F_c$  electron density map (blue) is contoured at  $1\sigma$  and the  $F_o - F_c$  map (green) is contoured at  $3\sigma$ . OMIT maps were generated as in **Figure S3**. Water molecules are shown as red spheres. Hydrogen bonds are shown as dashed yellow lines. (b) Structural alignment of ScLDM6×His I471T in complex with ITC (ITC with C atoms in yellow, haem with C atoms in magenta) and wild-type ScLDM6×His in complex with ITC (ITC and haem in cyan) (PDB ID: 5EQB). The position of ITC and the heme is unchanged between the ScLDM6×His wild-type and I471T mutant structures.

## References:

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