**Supplementary Information**

**Figure S1 | Chemical structures relevant to the vitamin K cycle.**

**a,** Vitamin K cycle involving γ-glutamyl carboxylase (GGCX) and vitamin K epoxide reductase (VKOR). Gla, γ-carboxylated Glu residues. **b**, Structure of the VKOR inhibitor warfarin and of ubiquinone.

**Figure S2 | Sequence alignment of VKORs from different species.**

VKORs from representative organisms were aligned by the CLUSTAL W2 algorithm [32] and manually optimized. Sequences were truncated after TM4. Conserved residues are shown in color, with the degree of conservation indicated by the color intensity. Cysteines are shown in red, arginines and lysines in pink, glutamines and asparagines in cyan, serine and threonine in green, prolines in yellow, glutamates and aspartates in purple, and hydrophobic residues in blue. Universally conserved residues are indicated by red stars. TM segments in the *Synechococcus sp.* structure are indicated by cylinders below the sequence. The 1/2-segment and 1/2-helix are shown in red. Residues whose mutation causes warfarin resistance in mammalian VKOR are indicated by crosses.

**Figure S3 | Stereo view of the fitted model and the experimental electron density map.**

The experimental map is contoured at 1σ, after B-factor (-80 $Å^2$) sharpening and solvent-flattening.

**Figure S4 | Packing of the *Synechococcus sp.* VKOR protein in the crystals.**

**a**, View down the x,y-plane of the unit cell (boxed area). Note that in this view the packing of the TM segments of VKOR resembles a type I membrane protein crystal. **b**, View down the z-axis of the unit cell. This corresponds to a view down the six-fold symmetry axis (see arrow in **a**). In this view, the packing resembles a type II membrane protein crystal.

**Figure S5 | Position of heavy metal ions, large amino acids, and methionines.**

**a**, The positions of heavy atoms that were detected after soaking the crystals with different compounds are indicated (Hg1, ethylmercurichthiosalicylic acid; Hg2, methylmercury chloride; Hg3, p-chloromercuribenzoic acid; Pt1, $K_2PtCl_4$). All heavy atom positions were determined by difference Fourier using model phases and showed density when contoured at 8$\sigma$ or higher. Note that all mercury compounds are bound specifically to cysteines and histidines, and the $K_2PtCl_4$ bound specifically to a methionine. Aromatic amino acids, which were used as anchor points during model building, are also indicated. The Trx-like domain is shown in blue, VKOR in pink, TM5 in light pink, and the loop between TM1 and 2 in red. **b,** The positions of all methionines, except that at the N-terminus, could be identified from X-ray data obtained with seleno-methionine substituted protein. The selenium atoms (in green) were located in an anomalous difference Fourier map, generated from phases that did not include the seleno-methionine data. The peak heights in the difference map are indicated.

**Figure S6 | Electron density map derived for the isolated Trx-like domain.**

**a**, Shown is a segment of the experimental electron density map derived from crystals of the isolated Trx-like domain at 1.5Å resolution and contoured at 1$\sigma$. The modeled amino acids are shown in stick presentation. **b**, The quality of the density map is illustrated by the fit of two amino acids.

**Figure S7 | Interaction of the 1/2-segment of VKOR with the Trx-like domain.**

The electrostatic surface of the pocket of the Trx-like domain is shown together with the bound peptide of the 1/2-segment of VKOR in ball and stick representation. Hydrophobic, basic, and acidic regions are colored in grey, blue, and red, respectively.

**Figure S8 | Stereo view of the active site of VKOR.**

The active site includes the cysteines of the CXXC motif in TM4 and the ubiquinone molecule (UQ). The experimental electron density map is contoured at 1σ (blue) and 3.5σ (red).

**Figure S9 | A model for the mammalian VKOR in complex with its postulated redox partner PDI.**

**a**, The structure of yeast PDI (in blue; pdb accession number 2B5E) [33] was superimposed on that of the Trx-like domain of the *Synechococcus sp.* VKOR protein. Yeast PDI has four Trx-like domains, but only the a-domain can be superimposed without other parts significantly clashing with the membrane (broken lines indicate the membrane boundaries). The figure therefore shows the a-domain of PDI replacing the *Synechococcus sp.* Trx-like domain. The VKOR part of the *Synechococcus sp.* protein (in pink) is shown without TM5 and the linker to the Trx-domain, as these are lacking in mammalian homologs. **b**, Superposition of the a-domain of yeast PDI and the *Synechococcus sp.* Trx-like domain. Note that both the CXXC motifs and the structural disulfide bridges (Cys231-Cys244 in *Synechococcus*) are well aligned.

**Figure S10 | Inhibition of the *Synechococcus sp.* VKOR homolog by a warfarin analog.**

**a**, Chemical structures of warfarin and of its close analog ferulenol.
**b**, The reduction of vitamin K was followed in a fluorometer in the presence of increasing concentrations of ferulenol. The data are normalized to the activity observed in the absence of inhibitor.

**Movie S1 | Overall structure.**

Overall structure of the protein, consisting of VKOR (pink) and the thioredoxin (Trx)-like domain (blue). The loop between TMs 1 and 2, containing the 1/2-segment and 1/2-helix, is shown in red. Ubiquinone (UQ) is shown in stick presentation. Sulfur atoms in cysteines are indicated in green.

**Movie S2 | The quinone and CXXC motif in VKOR.**

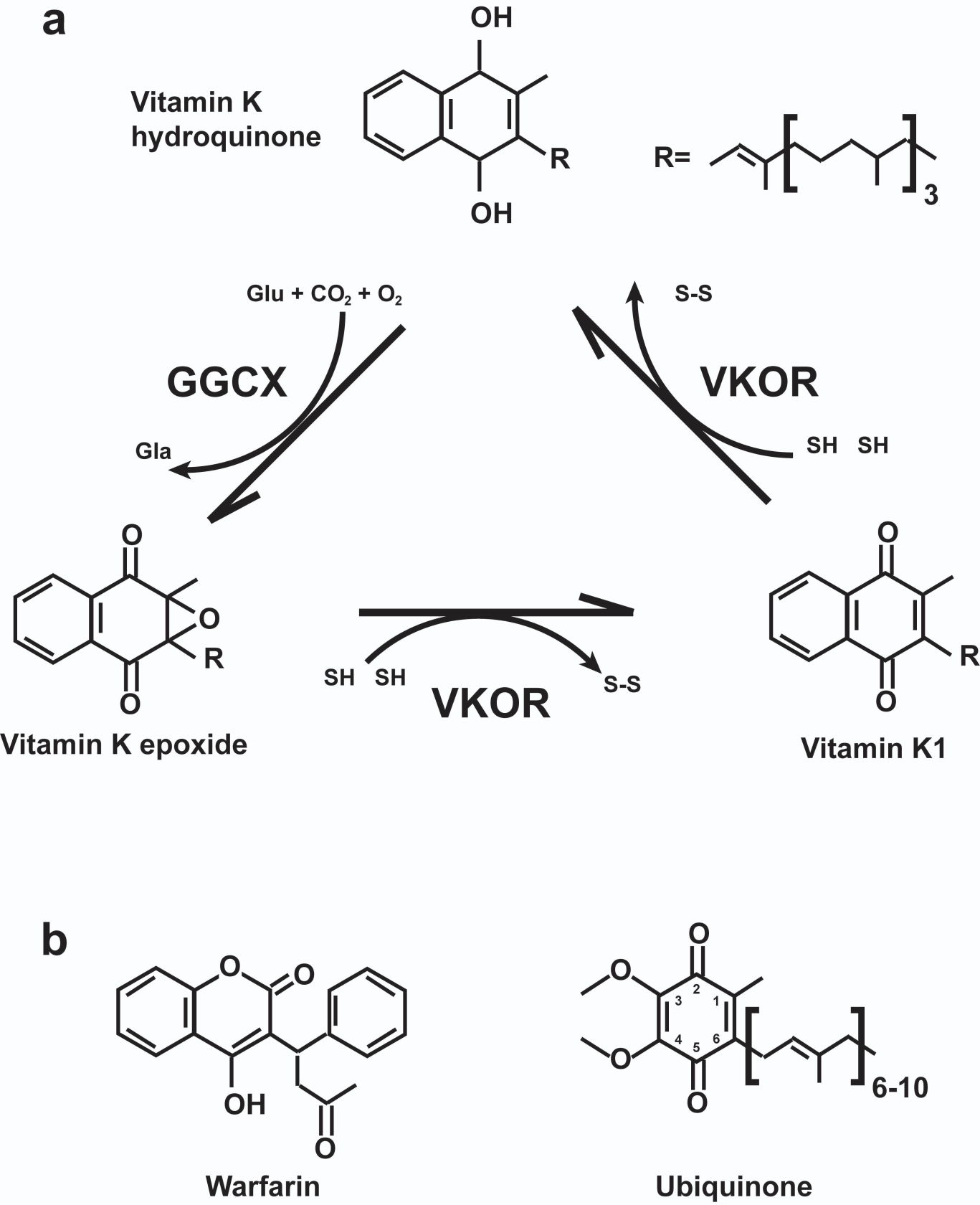
The active site of VKOR, including the cysteines of the CXXC motif in TM4 and the ubiquinone molecule (UQ), is shown together with the experimental electron density map. The map is initially contoured at 1σ (blue). Later in the movie, the contour level is increased to 4σ.

Table S1 Data collection, phasing and refinement statistics*

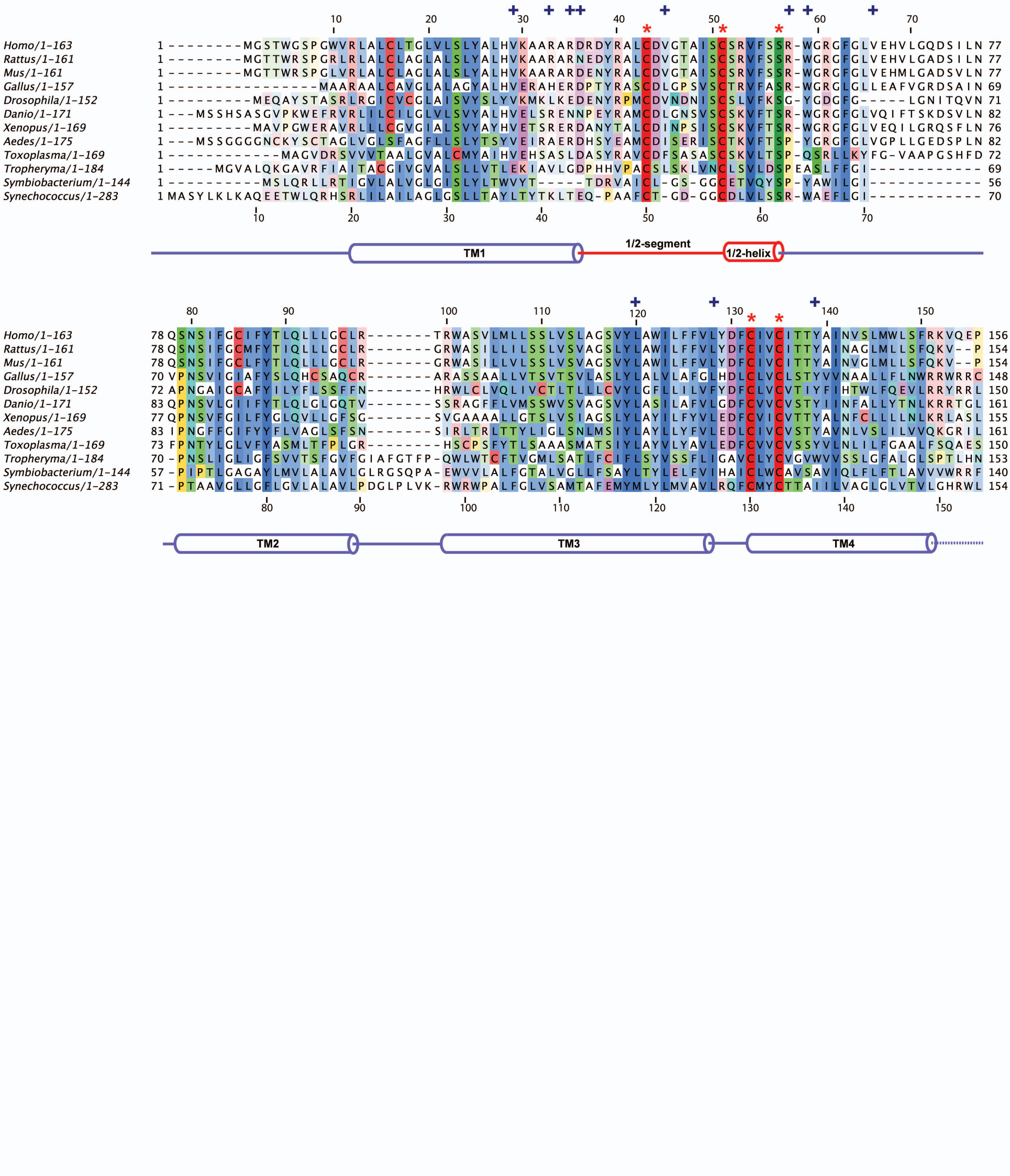
| | Full-length (VKOR-Trx) | Trx-like domain |
|---|---|---|
| **Data collection** | | |
| Space group | $P6_1$ | $P4_22_12_1$ |
| Cell dimensions | | |
| *a*, *b*, *c* (Å) | 137.0, 137.0, 68.5 | 57.2, 57.2, 58.1 |
| $\alpha, \beta, \gamma$ (°) | 90, 90, 120 | 90, 90, 90 |
| Resolution (Å) | 50-3.6 | 50-1.7 |
| $R_{sym}$ or $R_{merge}$ | 0.077 (0.725) | 0.058 (0.347) |
| $I/\sigma I$ | 13.9 (1.5) | 39.4 (9.4) |
| Completeness (%) | 99.1 (99.5) | 99.9 (100.0) |
| Redundancy | 3.1 (3.0) | 13.8 (14.1) |
| | | |
| **Refinement** | | |
| Resolution (Å) | 50-3.6 | 50-1.7 |
| No. reflections | 8,572 | 11,819 |
| $R_{work/}$ $R_{free}$ | 25.2/ 30.8 | 19.4/ 21.2 |
| No. atoms | | |
| Protein | 1991 | 714 |
| Ligand/ion | 33 | 0 |
| Water | 0 | 53 |
| B-factors | | |
| Protein | 53.6 | 19.5 |
| Ligand/ion | 104.3 | N/A |
| Water | N/A | 27.7 |
| R.m.s deviations | | |
| Bond lengths (Å) | 0.015 | 0.009 |
| Bond angles (°) | 1.802 | 1.749 |

* In each case, the data were collected from one crystal.

# Figure S1


a
Vitamin K hydroquinone
OH
R
OH
R= 3
Glu + CO2 + O2
GGCX
Gla
S-S
VKOR
SH SH
O
O
R
O
SH SH
VKOR
S-S
O
R
O
Vitamin K epoxide
Vitamin K1
b
O
O
OH
O
Warfarin
O
O
O
2
3
1
4
5
6
O
6-10
Ubiquinone

# Figure S2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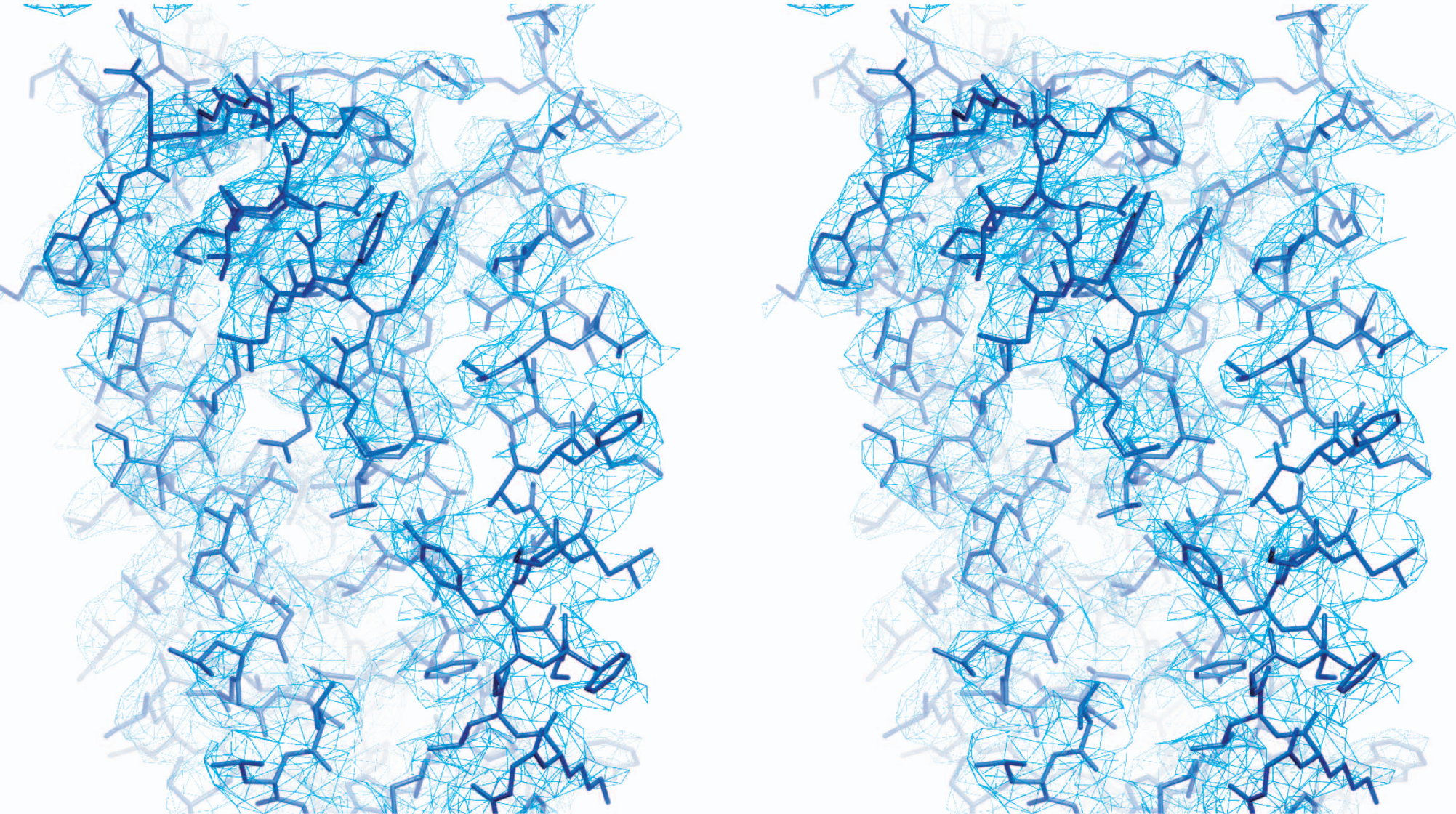
Rattus/1-161
Mus/1-161
Gallus/1-157
Drosophila/1-152
Danio/1-171
Xenopus/1-169
Aedes/1-175
Toxoplasma/1-169
Tropheryma/1-184
Symbiobacterium/1-144
Synechococcus/1-283
TM1
1/2-segment
1/2-helix
TM2
TM3
TM4

# Figure S3



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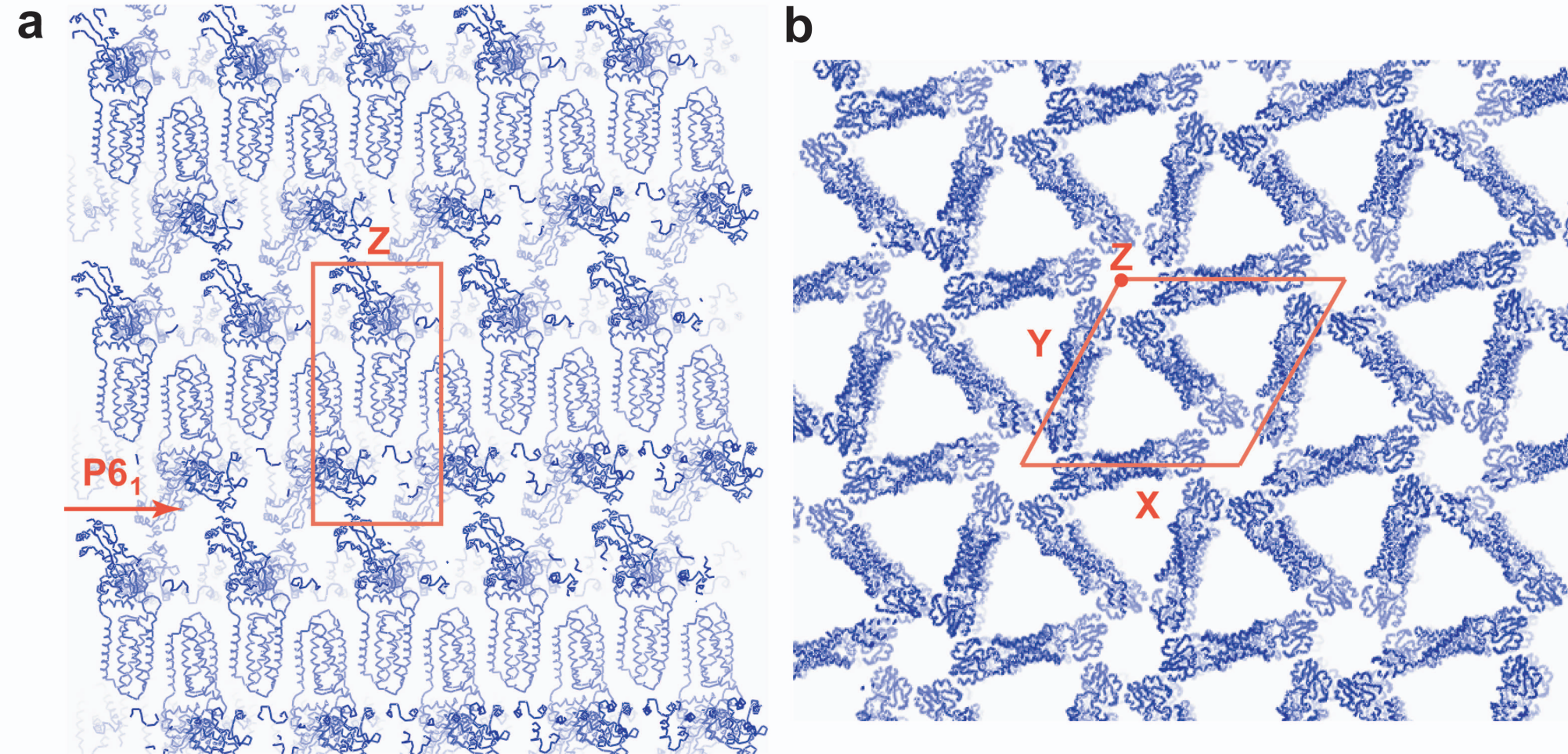

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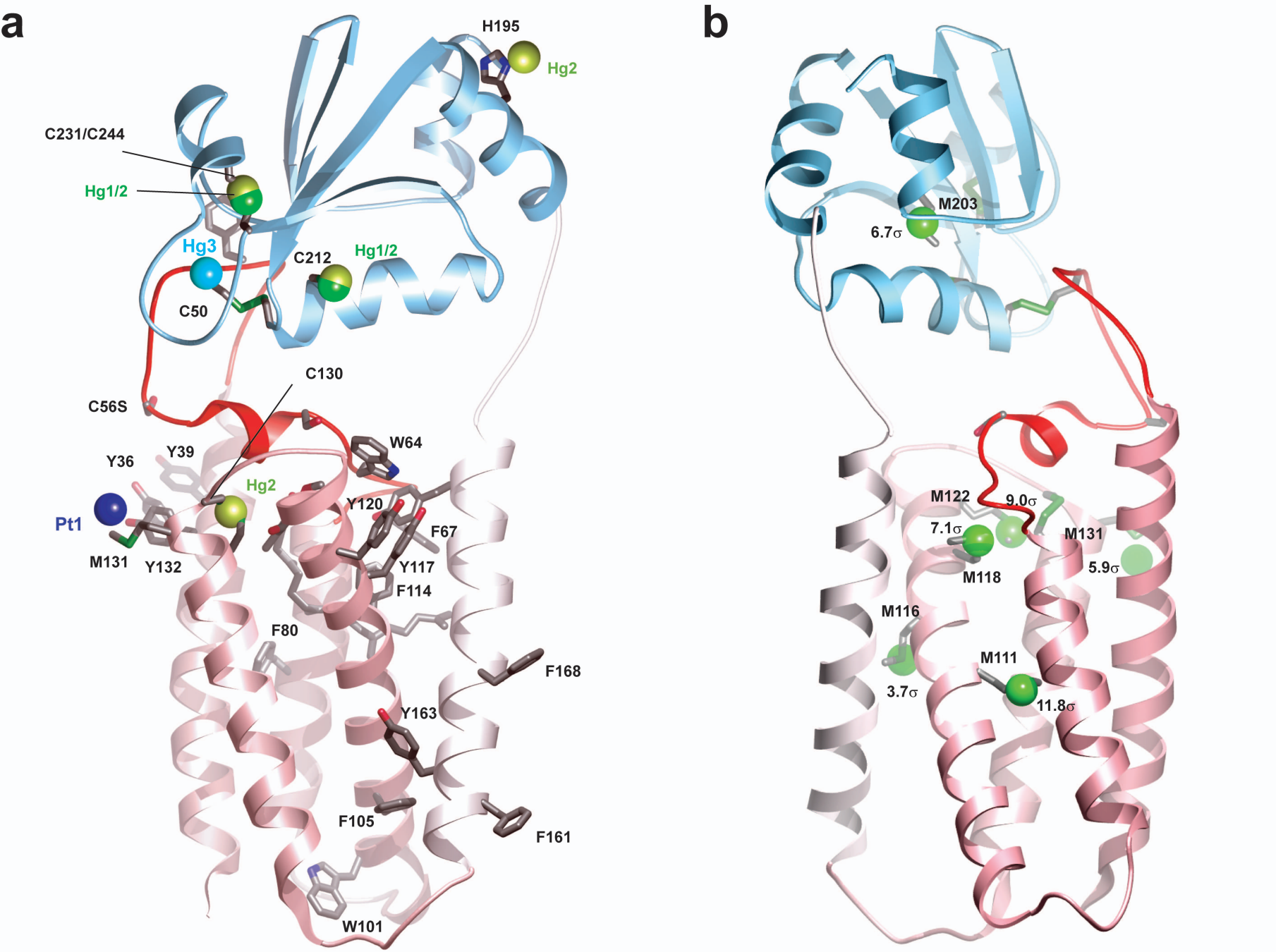
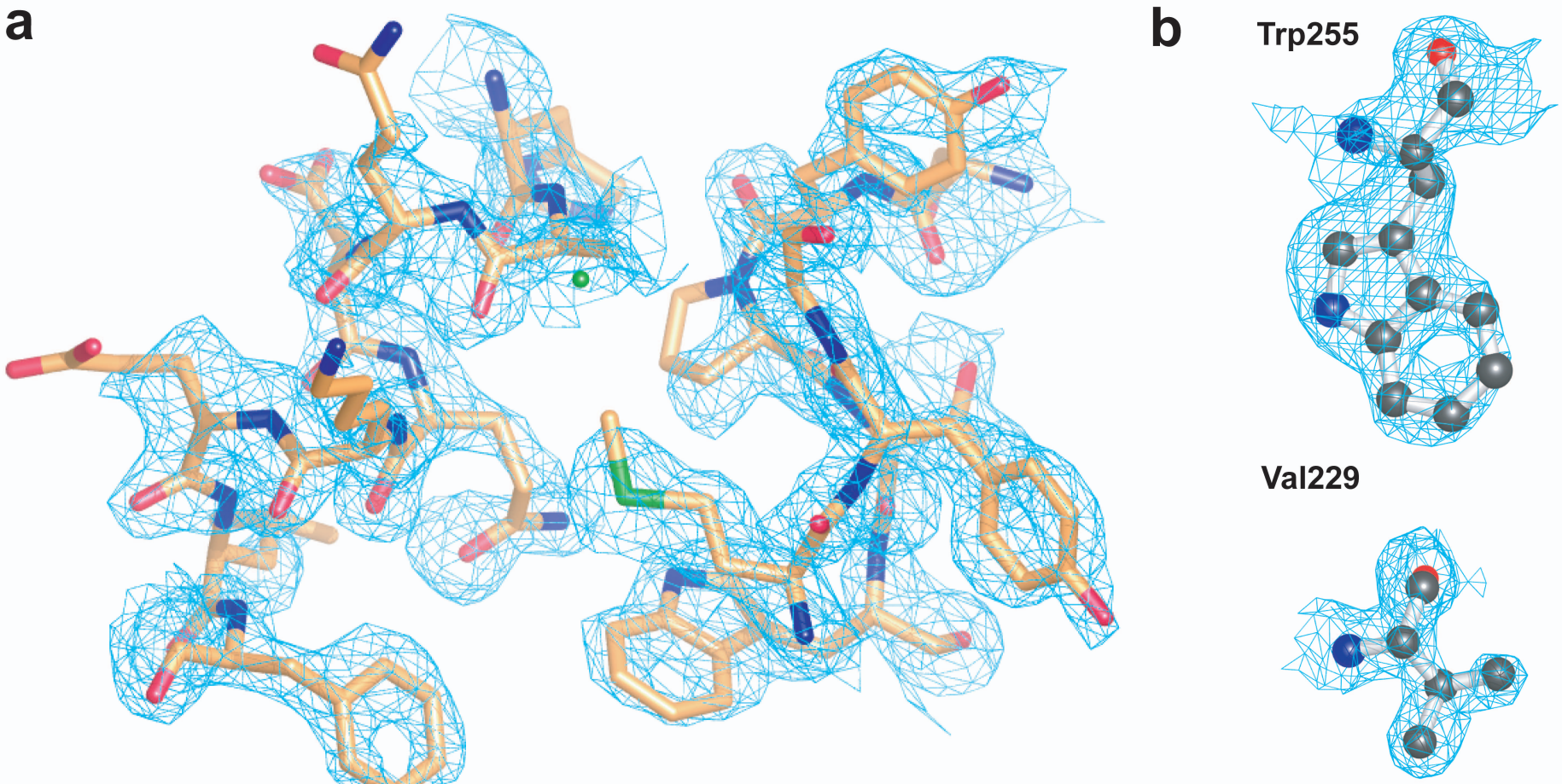

a
H195
Hg2
C231/C244
Hg1/2
Hg3
C212
Hg1/2
C50
C130
C56S
W64
Y36
Y39
Hg2
Pt1
Y120
F67
M131
Y132
Y117
F114
F80
F168
Y163
F105
F161
W101
b
M203
6.7σ
M122
9.0σ
7.1σ
M131
5.9σ
M118
M116
M111
3.7σ
11.8σ

# Figure S6



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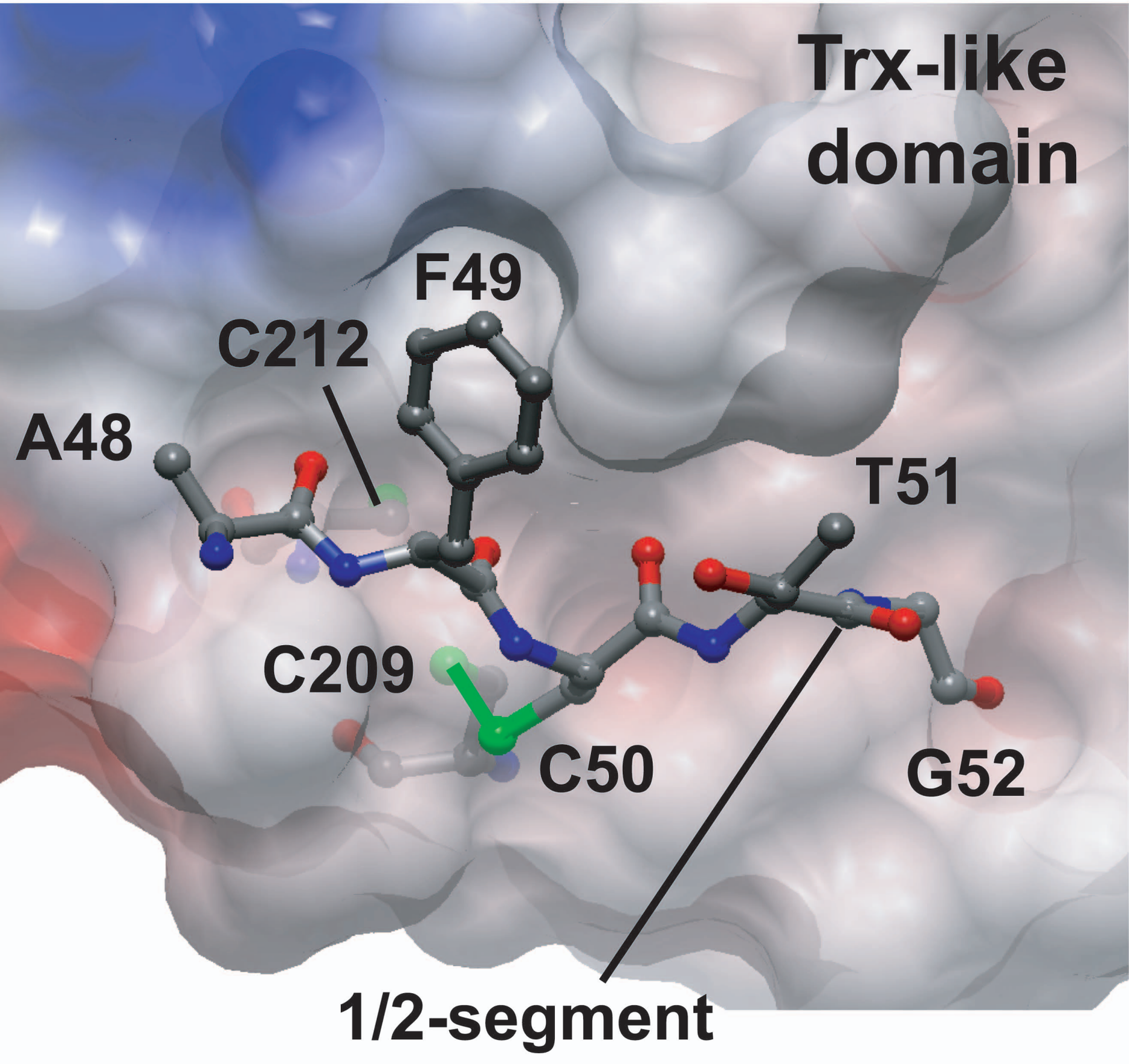
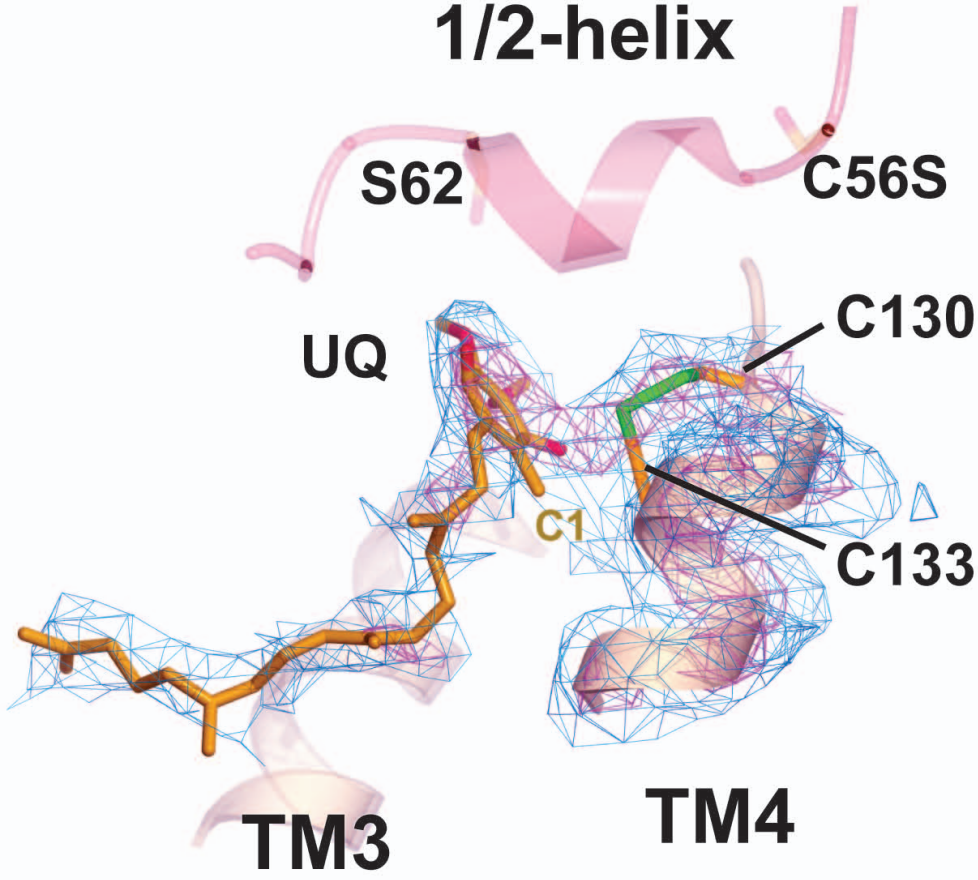
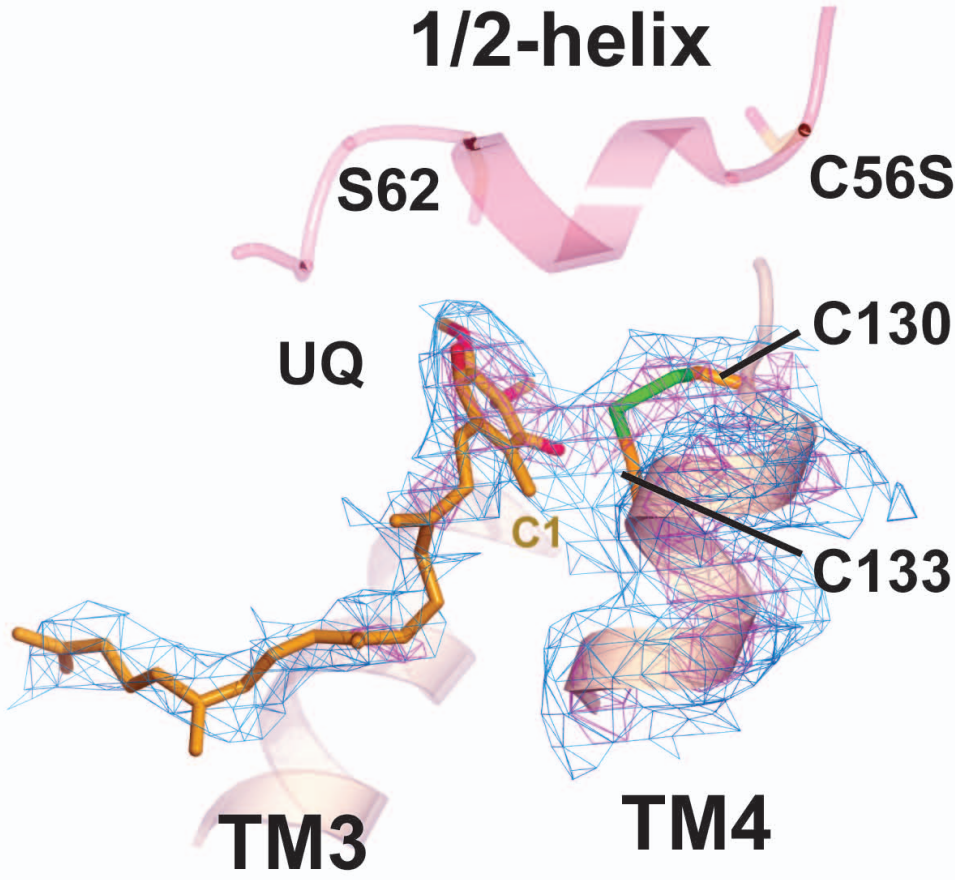
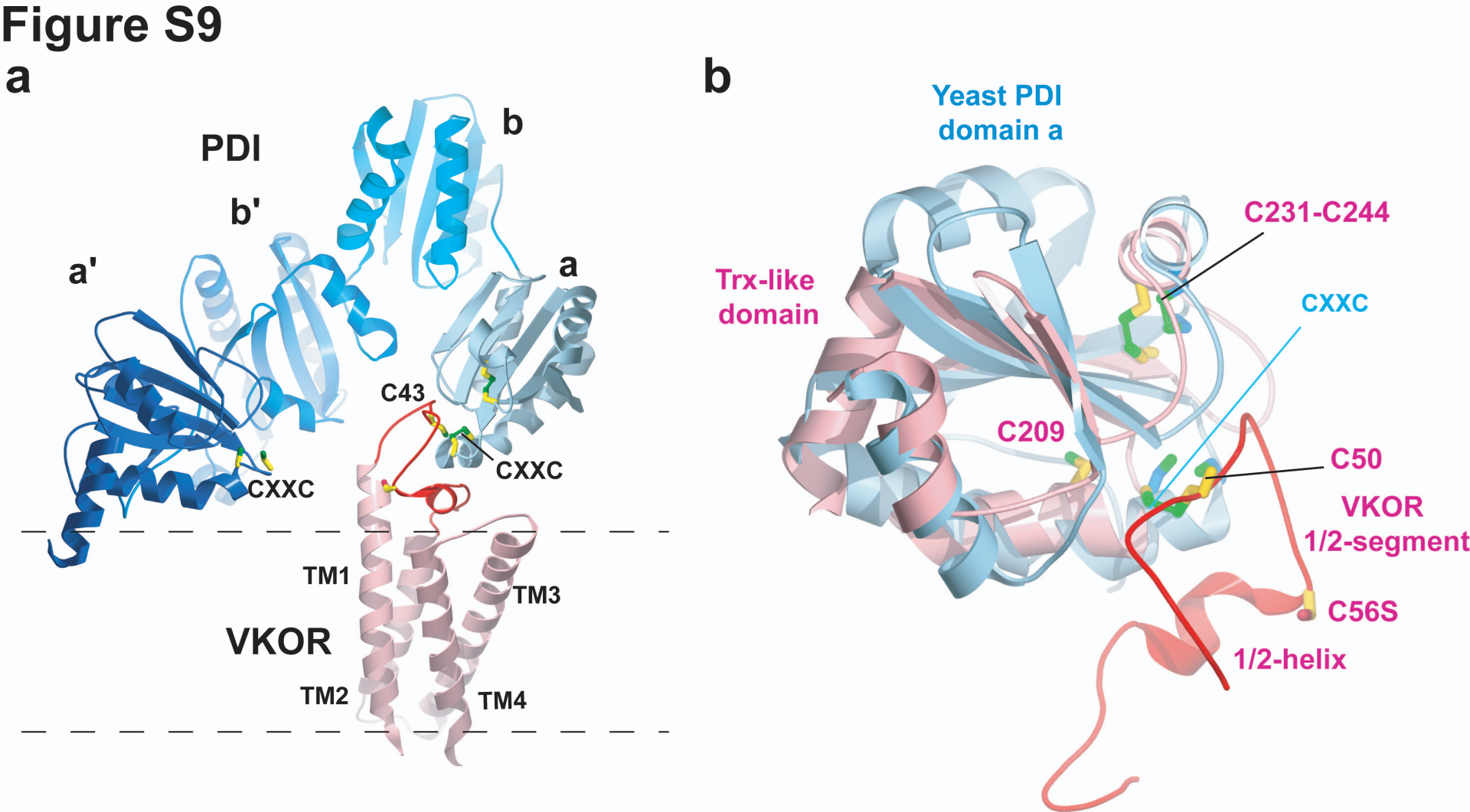


# Figure S8


1/2-helix
S62
C56S
UQ
C130
C1
C133
TM3
TM4

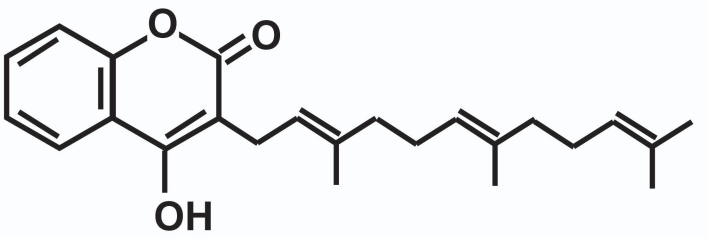

1/2-helix
S62
C56S
UQ
C130
C1
C133
TM3
TM4

# Figure S9

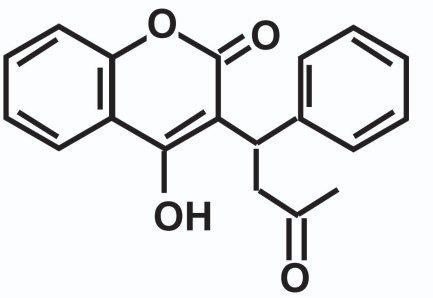

a
PDI
b
b'
a'
a
C43
CXXC
CXXC
TM1
TM3
VKOR
TM2
TM4
b
Yeast PDI
domain a
C231-C244
Trx-like
domain
CXXC
C209
C50
VKOR
1/2-segment
C56S
1/2-helix

# Figure S10

a




Ferulenol




Warfarin

b