

Supplemental Material

Additional PfCRT Mutations Driven By Selective Pressure for Improved Fitness Can Result in the Loss of Piperaquine Resistance and Altered *Plasmodium falciparum* Physiology

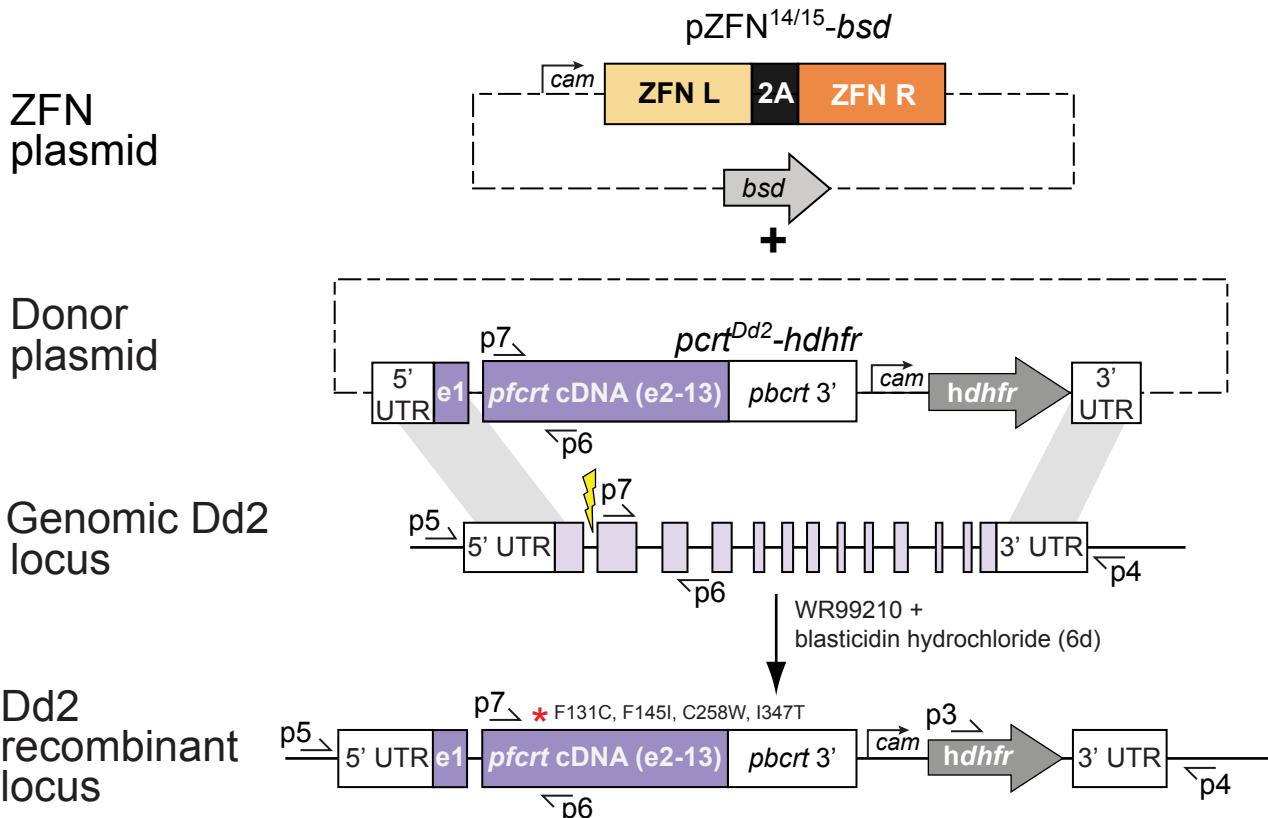
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Figure S1

A



B

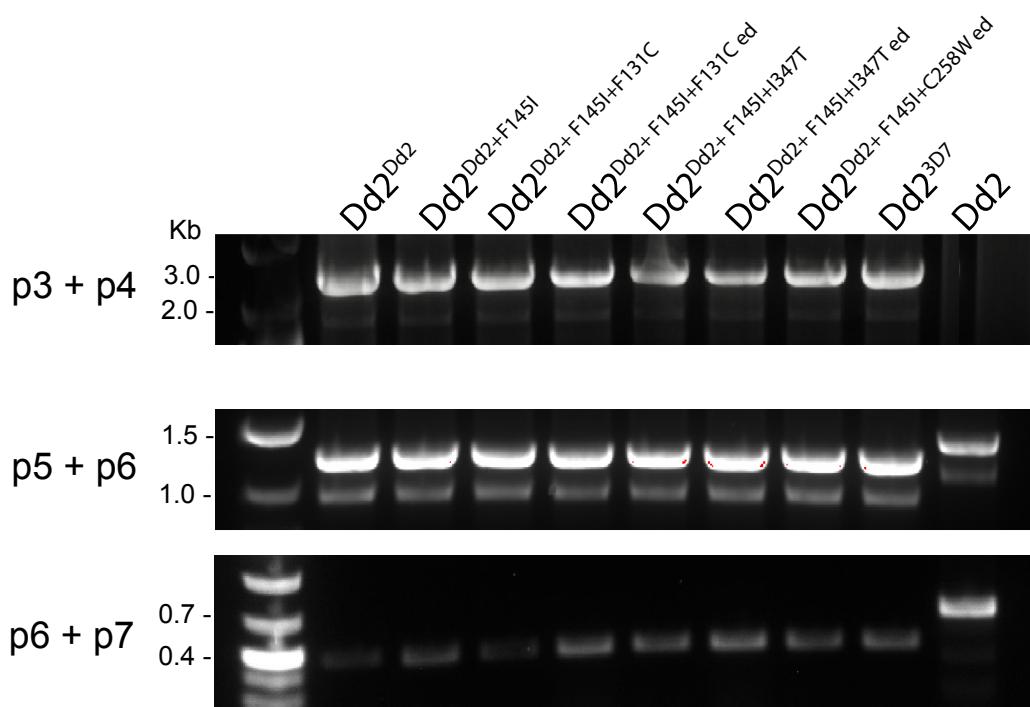


Fig. S1. Zinc-finger nuclease (ZFN)-mediated editing of *pfCRT*. (A) The *pfCRT* gene was edited using a two-plasmid approach, one containing the homologous donor template and the other containing the *pfCRT*-specific pair of ZFNs linked by a 2A ribosome skip peptide (1). Parasites were selected for the human dihydrofolate reductase (*hdhfr*) and the blasticidin S-deaminase (BSD) markers with WR99210 and blasticidin hydrochloride, respectively. (B) Three sets of PCRs were performed to confirm editing and the modified locus was verified by Sanger sequencing. Primer locations are denoted in (A), and sequences are listed in **Table S7**.

Figure S2

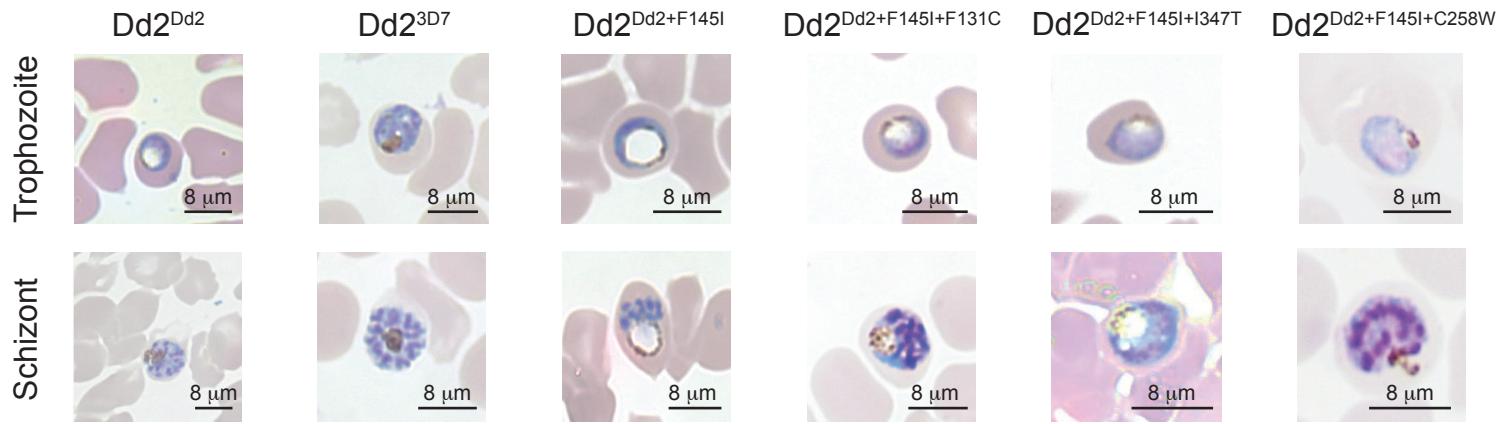


Fig. S2. Cell morphology of *pfCRT*-edited parasites. Dd2^{Dd2+F145I} has distended digestive vacuoles characteristic of piperaquine-resistant parasites in both the trophozoite and schizont asexual blood stages. Dd2^{Dd2+F145I+F131C}, Dd2^{Dd2+F145I+I347T}, and Dd2^{Dd2+F145I+C258W} vacuoles appear smaller compared to Dd2^{Dd2+F145I}. Note that the partially bloated vacuoles evident in Dd2^{Dd2+F145I+F131C} and Dd2^{Dd2+F145I+I347T} associate with a minimal change in peptide accumulation relative to Dd2^{Dd2+F145I} (Fig. 4).

Figure S3

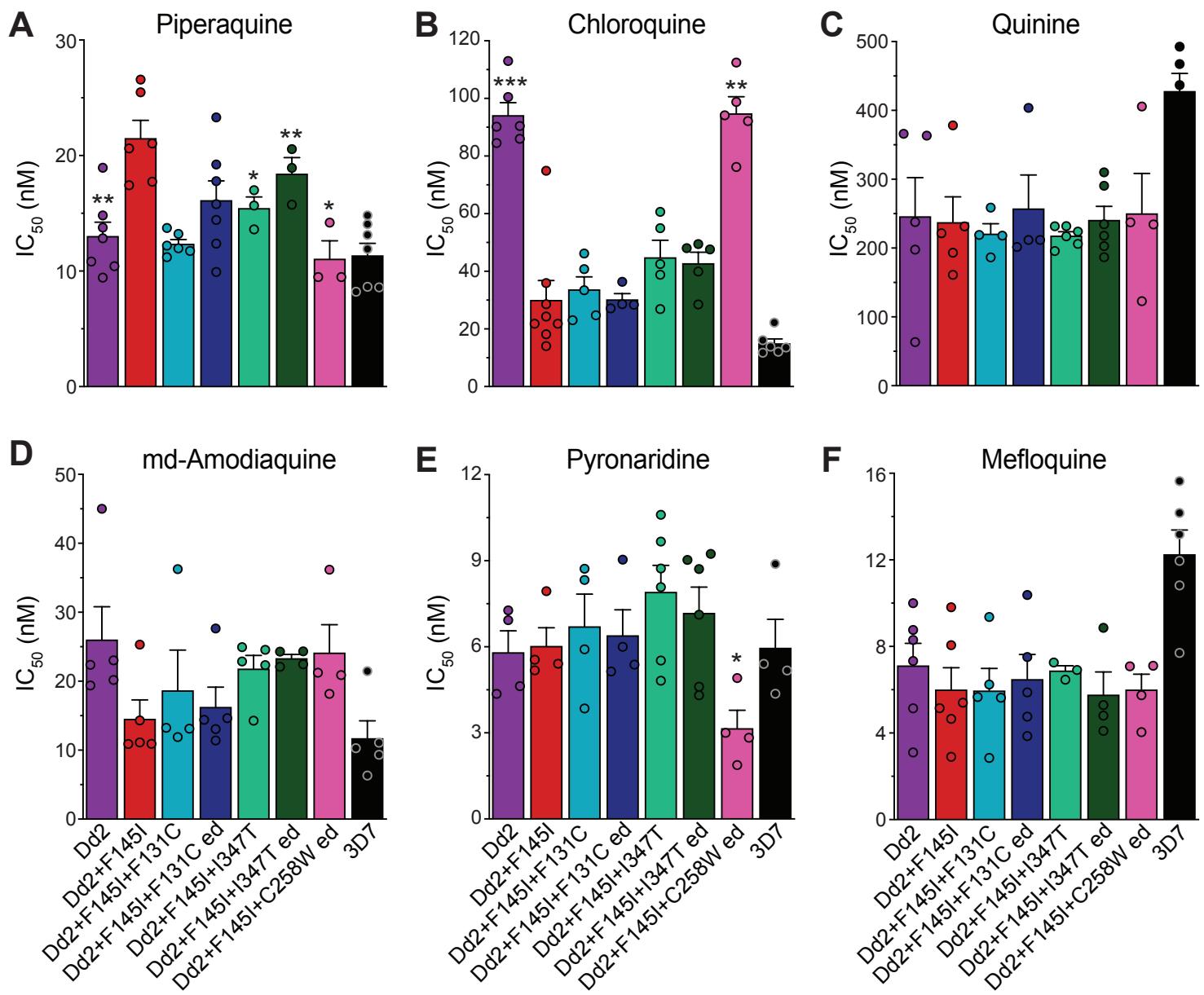


Fig. S3. IC_{50} values for relevant antimalarials. Mean \pm SEM IC_{50} values (Table S2) were calculated from 72-hr dose-response assays for: (A) Piperaquine; (B) Chloroquine; (C) Quinine; (D) monodesethyl (md)-Amodiaquine; (E) Pyronaridine; and (F) Mefloquine. N, n = 4-7, 2. Statistical significance was determined using Mann-Whitney U tests as compared to the isogenic Dd2^{Dd2+F145I} line. *P < 0.05; **P < 0.01; ***P < 0.001. Individual circles indicate values from each independent experiment.

Figure S4

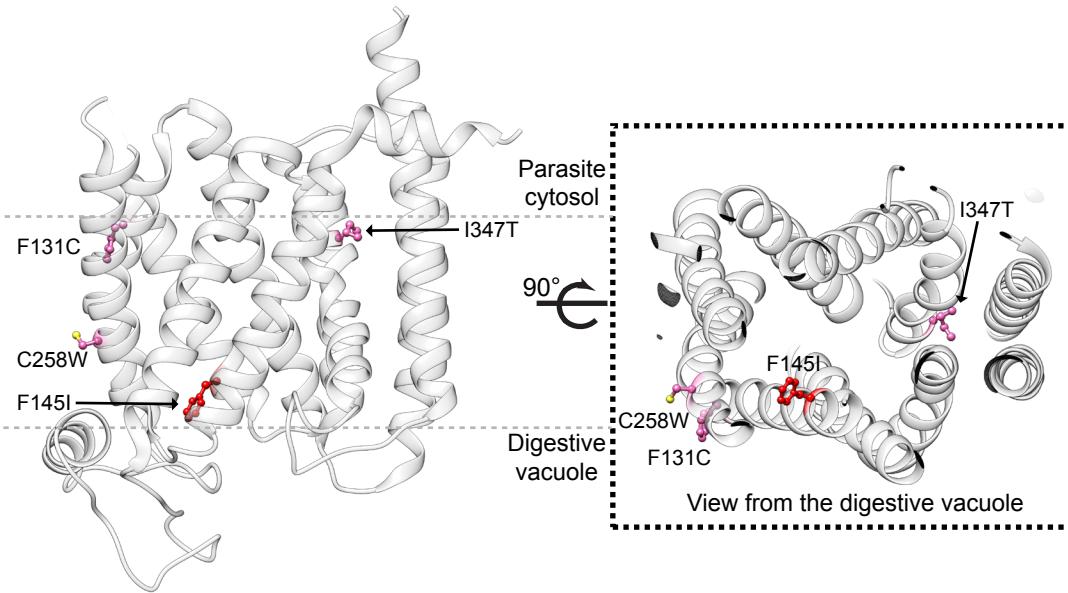


Fig. S4. Mapping of mutations onto the PfCRT structure. F145I, F131C, C258W, and I347T are mapped onto the known 7G8 cryo-EM structure (2). Mutations have their side chains rendered as sticks and are colored in red (F145I) or pink (F131C, C258W and I347T). The remaining structures are rendered in cartoon and colored in white. Views are shown vertically (digestive vacuole to the bottom) and rotated to show the structure from the digestive vacuole side.

Figure S5

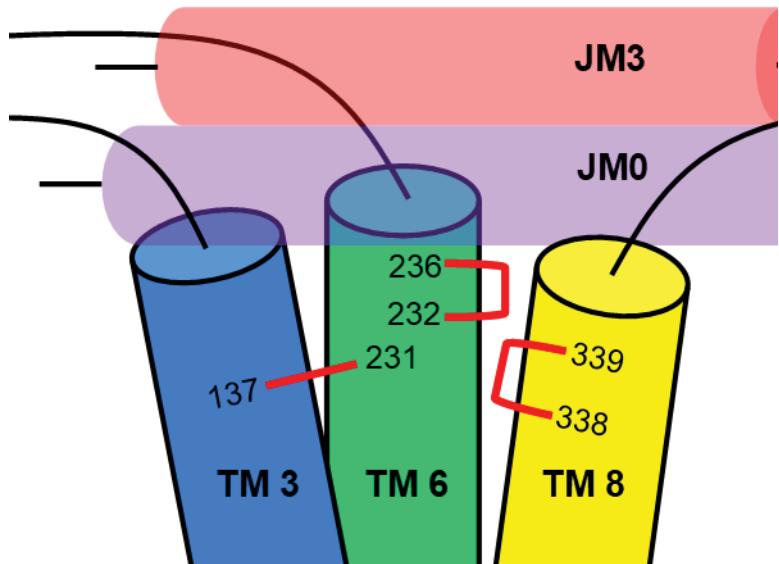
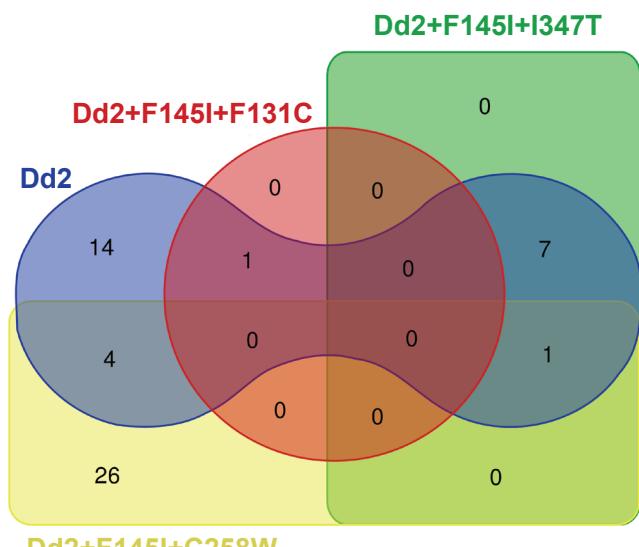


Fig. S5. Visual representation of salt bridge interactions broken as Dd2 PfCRT evolves to any of the F145I mutant isoforms. Transmembrane (TM) helix numbers are denoted, with PfCRT “zipper” bundled helices JM3 and JM0 shown above [3]. The full list off salt bridge interactions is listed in **Table S4**.

Figure S6



Dd2+F145I+C258W

Fig. S6. Venn diagram of numbers of significantly altered peptides. Venn diagram showing numbers of peptides whose levels were significantly increased or decreased peptides for the lines Dd2^{Dd2} (blue), Dd2^{Dd2+F145I+F131C} (red), Dd2^{Dd2+F145I+I347T} (green), or Dd2^{Dd2+F145I+C258W} (yellow), relative to the isogenic Dd2^{Dd2+F145I} line. The largest numbers of differences were observed with Dd2^{Dd2+F145I+C258W} and Dd2^{Dd2}, as reflected in the heatmap (**Fig. 4A**) that showed these two lines being the most divergent compared with Dd2^{Dd2+F145I}. Significance was attributed to a peptide when it showed a statistically significant difference ($P < 0.05$) between the Dd2^{Dd2+F145I} reference line and an isogenic test line (Student's *t* test, data obtained from 3 independent experiments; see **Table S6**).

Figure S7

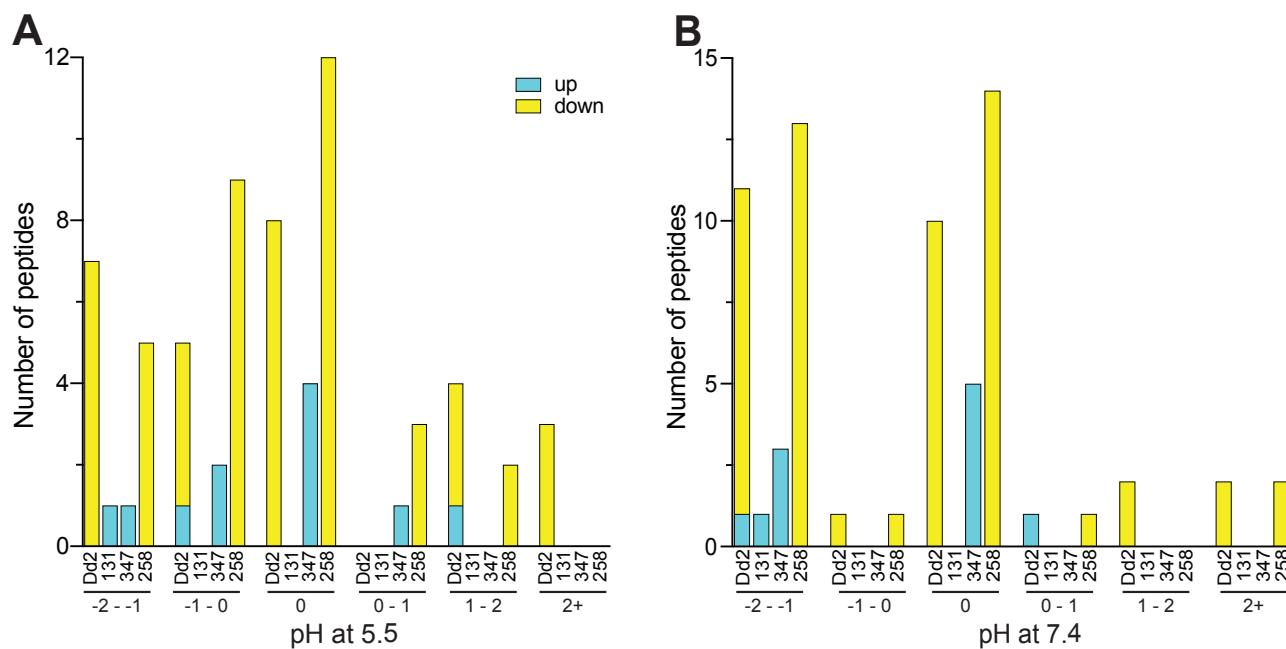


Fig. S7. Number of differentially accumulated peptides shown as a function of peptide charge of accumulated peptides at pH 5.5 or 7.4. Plots show differences in peptide levels between lines with a given mutation and Dd2^{Dd2+F145I}. Peptides are classified by (A) charge at pH 5.5 (representing the DV lumen) or (B) charge at pH 7.4 (representing the cytosol). Details are provided in **Table S6**.

Figure S8

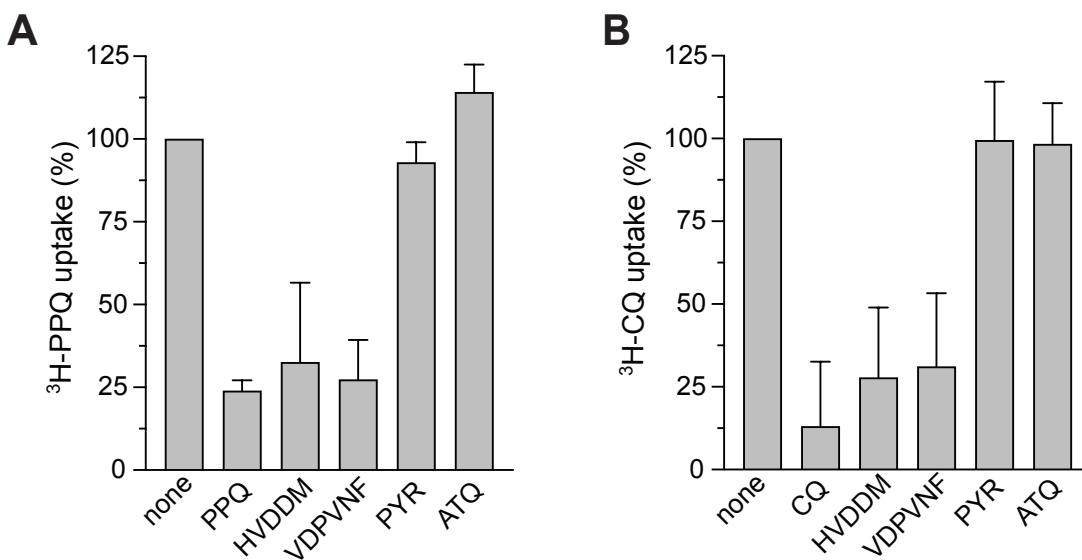


Fig. S8. Peptides HVDDM and VDPVNF inhibit ${}^3\text{H}$ -PPQ and ${}^3\text{H}$ -CQ transport via PfCRT. Uptake of (A) 50 nM ${}^3\text{H}$ -PPQ by 7G8+F145I PfCRT or (B) ${}^3\text{H}$ -CQ by 7G8 PfCRT was measured for 1 min in the presence or absence of the 25 μM HVDDM or VDPVNF. Values were normalized to the signal in the absence of the non-radiolabelled compound ("none"). CQ, PPQ, and the non-PfCRT related drugs pyrimethamine (PYR) and atovaquone (ATQ) were also tested at 25 μM . Data are means \pm s.d. of 3 independent experiments.

Table S1 Piperaquine survival assay values of *pfcrt*-modified parasite lines.

	Dd2 ^{Dd2}	Dd2 ^{3D7}	Dd2 ^{Dd2+F145I}	Dd2 ^{Dd2+F145I+F131C}	Dd2 ^{Dd2+F145I+F131C (edited)}	Dd2 ^{Dd2+F145I+I347T}	Dd2 ^{Dd2+F145I+I347T (edited)}	Dd2 ^{Dd2+F145I+C258W (edited)}
1600 nM	3.5 ± 0.7	2.9 ± 1.5	35.0 ± 2.0	28.4 ± 3.6	33.6 ± 1.0	6.7 ± 1.1	13.3 ± 1.9	3.2 ± 0.6
N	4	6	7	7	8	3	2	3
P vs Dd2 ^{Dd2}	—	0.61	0.006	0.006	0.010	0.11	0.13	>0.99
P vs Dd2 ^{Dd2+F145I}	0.006	—	—	0.24	0.51	0.056	0.017	0.017
800 nM	3.1 ± 1.1	3.3 ± 0.5	32.9 ± 3.0	28.5 ± 2.9	30.5 ± 2.0	2.9 ± 1.5	5.9 ± 4.5	3.3 ± 0.7
N	4	6	7	7	7	3	2	3
P vs Dd2 ^{Dd2}	—	>0.99	0.006	0.006	0.006	0.86	0.80	0.86
P vs Dd2 ^{Dd2+F145I}	0.006	—	—	0.30	0.59	0.017	0.056	0.017
400 nM	3.3 ± 0.8	2.5 ± 0.5	23.1 ± 2.4	18.8 ± 3.2	19.2 ± 2.3	1.9 ± 0.3	2.9 ± 0.6	3.3 ± 0.7
N	4	6	7	7	8	3	2	3
P vs Dd2 ^{Dd2}	—	0.35	0.010	0.010	0.006	0.40	0.80	>0.99
P vs Dd2 ^{Dd2+F145I}	0.010	—	—	0.12	0.22	0.024	0.071	0.024
200 nM	3.8 ± 0.8	2.8 ± 0.6	18.4 ± 3.4	14.2 ± 2.4	12.1 ± 1.7	1.7 ± 0.2	3.2 ± 0.7	4.3 ± 1.1
N	4	6	6	7	8	3	2	3
P vs Dd2 ^{Dd2}	—	0.26	0.010	0.006	0.016	0.40	0.53	>0.99
P vs Dd2 ^{Dd2+F145I}	0.010	—	—	0.17	0.23	0.024	0.071	0.024
100 nM	4.3 ± 0.9	2.8 ± 0.5	15.4 ± 2.4	14.2 ± 2.4	10.1 ± 1.3	1.4 ± 0.1	4.2 ± 0.9	4.5 ± 1.1
N	4	6	6	8	8	3	2	3
P vs Dd2 ^{Dd2}	—	0.26	0.010	0.004	0.038	0.057	0.53	>0.99
P vs Dd2 ^{Dd2+F145I}	0.010	—	—	0.47	0.12	0.024	0.071	0.024
50 nM	4.1 ± 0.6	2.4 ± 0.5	17.5 ± 2.6	18.6 ± 2.9	11.9 ± 3.0	1.6 ± 0.4	2.6 ± 0.7	26.3 ± 13.7
N	4	6	7	7	8	3	2	3
P vs Dd2 ^{Dd2}	—	0.038	0.005	0.010	0.010	0.057	0.13	0.057
P vs Dd2 ^{Dd2+F145I}	0.005	—	—	0.78	0.17	0.024	0.071	>0.99
25 nM	8.5 ± 1.5	5.0 ± 1.0	18.2 ± 4.3	28.8 ± 5.9	36.1 ± 7.2	22.2 ± 6.1	20.4 ± 11.4	50.8 ± 18.3
N	5	6	5	6	8	3	2	3
P vs Dd2 ^{Dd2}	—	0.13	0.016	0.009	0.008	0.38	0.14	0.036
P vs Dd2 ^{Dd2+F145I}	0.016	—	—	0.40	0.048	0.96	0.81	0.054
12.5 nM	28.8 ± 5.3	44.7 ± 1.1	53.8 ± 5.4	38.6 ± 7.1	67.1 ± 7.7	58.6 ± 9.8	58.0 ± 24.7	75.9 ± 15.8
N	4	6	7	6	4	3	2	3
P vs Dd2 ^{Dd2}	—	0.016	0.006	0.35	0.029	0.057	0.53	0.057
P vs Dd2 ^{Dd2+F145I}	0.006	—	—	0.35	0.15	0.95	>0.99	0.25
6.25 nM	58.6 ± 6.5	77.6 ± 3.0	69.2 ± 3.2	54.2 ± 6.3	79.2 ± 5.1	78.3 ± 4.4	70.6 ± 5.1	89.0 ± 4.0
N	4	5	7	6	8	3	2	3
P vs Dd2 ^{Dd2}	—	0.032	0.21	0.91	0.064	0.057	>0.99	0.057
P vs Dd2 ^{Dd2+F145I}	0.21	—	—	0.093	0.10	0.17	>0.99	0.033
3.125 nM	79.0 ± 3.0	89.9 ± 2.2	82.9 ± 2.9	69.7 ± 5.1	87.4 ± 3.0	92.6 ± 12.4	99.6 ± 0.0	91.5 ± 3.1
N	4	4	7	5	5	2	1	3
P vs Dd2 ^{Dd2}	—	0.057	0.39	0.11	0.19	0.53	—	0.11
P vs Dd2 ^{Dd2+F145I}	0.39	—	—	0.043	0.25	0.47	—	0.18

Piperaquine survival assay (PSA) values (nM) indicate the mean ± SEM, as determined in 2 to 8 independent assays performed in duplicate. Parasite survival is defined as the ratio of the parasitemias of the PPQ-treated to the no-drug control wells. This assay measures the survival of synchronous ring-stage parasites (0–6 hr post-invasion) exposed to PPQ for 72 hr, prior to measuring parasitemias by flow cytometry. N, number of independent assays. Statistical significance was determined via non-parametric Mann-Whitney U tests. P values are reported for comparisons with the parasite lines Dd2^{Dd2} and Dd2^{Dd2+F145I}.

*P < 0.05

**P < 0.01

Table S2 Mean IC₅₀ and IC₉₀ values of *pfcrt*-modified parasite lines.

	Dd2 ^{Dd2}	Dd2 ^{3D7}	Dd2 ^{Dd2+F145I}	Dd2 ^{Dd2+F145I+F131C}	Dd2 ^{Dd2+F145I+F131C (edited)}	Dd2 ^{Dd2+F145I+I347T}	Dd2 ^{Dd2+F145I+I347T (edited)}	Dd2 ^{Dd2+F145I+C258W (edited)}
PPQ IC₅₀ (nM)	13.0 ± 1.3	11.4 ± 1.1	21.5 ± 1.6	12.3 ± 0.4	16.1 ± 1.7	15.4 ± 1.0	18.4 ± 1.4	11.1 ± 1.5
N	7	7	6	6	7	3	3	3
P vs Dd2 ^{Dd2}	–	0.40	0.005	0.21	0.95	0.27	0.007	0.48
P vs Dd2 ^{Dd2+F145I}	0.005	–	–	0.002	0.073	0.024	0.26	0.024
PPQ IC₉₀ (nM)	23.7 ± 1.9	23.6 ± 3.5	5636 ± 690.9	3601 ± 236.6	2753 ± 225.9	28.1 ± 4.2	30.1 ± 3.3	22.6 ± 2.0
P vs Dd2 ^{Dd2}	–	0.96	0.006	0.017	0.017	0.27	0.022	>0.99
P vs Dd2 ^{Dd2+F145I}	0.006	0.004	–	0.057	0.057	0.016	0.010	0.032
CQ IC₅₀ (nM)	94.1 ± 4.4	14.9 ± 1.6	30.0 ± 6.9	33.6 ± 4.5	30.1 ± 2.1	44.8 ± 6.0	42.7 ± 3.9	94.7 ± 5.8
N	6	6	8	5	4	5	5	5
P vs Dd2 ^{Dd2}	–	0.002	0.001	0.004	0.010	0.004	0.004	0.792
P vs Dd2 ^{Dd2+F145I}	0.001	–	–	0.208	0.301	0.061	0.061	0.002
CQ IC₉₀ (nM)	172.2 ± 18.5	21.9 ± 3.0	63.1 ± 20.1	60.2 ± 10.4	65.4 ± 6.7	98.7 ± 8.7	103.7 ± 5.7	175.1 ± 22.7
P vs Dd2 ^{Dd2}	–	0.002	0.015	0.002	0.010	0.015	0.002	0.75
P vs Dd2 ^{Dd2+F145I}	0.015	–	–	0.31	0.41	0.15	0.15	0.032
md-CQ IC₅₀ (nM)	572.7 ± 77.6	21.1 ± 2.8	124.6 ± 19.6	165.7 ± 17.0	180.8 ± 20.6	205.8 ± 39.9	192.4 ± 26.8	840.8 ± 95.7
N	6	6	5	5	4	5	5	4
P vs Dd2 ^{Dd2}	–	0.002	0.004	0.004	0.004	0.004	0.004	0.083
P vs Dd2 ^{Dd2+F145I}	0.004	–	–	0.31	0.095	0.095	0.032	0.008
md-CQ IC₉₀ (nM)	1152 ± 143.2	347.9 ± 5.9	316.9 ± 69.9	397.4 ± 57.1	366.6 ± 41.5	600.2 ± 83.4	644.2 ± 68.3	1641 ± 86.4
P vs Dd2 ^{Dd2}	–	0.002	0.010	0.004	0.010	0.004	0.004	0.052
P vs Dd2 ^{Dd2+F145I}	0.010	–	–	0.29	0.49	0.11	0.032	0.016
md-ADQ IC₅₀ (nM)	26.0 ± 4.8	11.7 ± 2.6	14.5 ± 2.8	18.6 ± 5.9	16.2 ± 2.9	21.8 ± 1.9	23.3 ± 0.6	24.1 ± 4.1
N	5	5	5	4	5	5	4	4
P vs Dd2 ^{Dd2}	–	0.032	0.095	0.19	0.095	0.56	>0.99	0.75
P vs Dd2 ^{Dd2+F145I}	0.095	–	–	0.41	0.22	0.22	0.19	0.11
md-ADQ IC₉₀ (nM)	36.7 ± 7.3	16.9 ± 3.1	25.7 ± 7.2	32.9 ± 8.3	31.6 ± 6.1	38.9 ± 4.4	37.1 ± 4.4	36.9 ± 7.1
P vs Dd2 ^{Dd2}	–	0.016	0.087	0.19	0.15	0.69	>0.99	0.73
P vs Dd2 ^{Dd2+F145I}	0.087	–	–	0.37	0.21	0.21	0.21	0.13
QN IC₅₀ (nM)	245.7 ± 56.5	427.7 ± 26.1	237.1 ± 37.4	220.6 ± 14.8	257.2 ± 48.9	217.7 ± 5.9	240.4 ± 20.1	257.2 ± 48.9
N	5	5	5	4	4	6	6	4
P vs Dd2 ^{Dd2}	–	0.032	0.84	0.73	0.90	0.54	0.79	>0.99
P vs Dd2 ^{Dd2+F145I}	0.84	–	–	0.90	0.90	0.79	0.79	0.56
QN IC₉₀ (nM)	745.0 ± 113.2	923.2 ± 31.7	652.5 ± 75.4	709.7 ± 55.4	623.5 ± 45.7	637.0 ± 73.7	663.8 ± 34.3	n.d.
P vs Dd2 ^{Dd2}	–	0.41	0.49	0.89	0.86	0.26	0.76	–
P vs Dd2 ^{Dd2+F145I}	0.49	–	–	0.49	0.23	0.76	0.91	–
PND IC₅₀ (nM)	5.8 ± 0.8	6.0 ± 1.0	6.0 ± 0.6	6.7 ± 1.1	6.4 ± 0.9	7.9 ± 0.9	7.2 ± 0.9	3.2 ± 0.6
N	4	4	4	4	4	6	6	4
P vs Dd2 ^{Dd2}	–	0.89	0.69	0.69	0.69	0.11	0.48	0.11
P vs Dd2 ^{Dd2+F145I}	0.69	–	–	0.49	>0.99	0.25	0.54	0.029
PND IC₉₀ (nM)	9.4 ± 1.3	9.6 ± 1.3	11.4 ± 0.4	12.3 ± 0.9	12.3 ± 2.2	14.4 ± 2.4	9.4 ± 1.4	6.4 ± 1.6
P vs Dd2 ^{Dd2}	–	>0.99	0.11	0.11	0.63	0.20	0.51	0.11
P vs Dd2 ^{Dd2+F145I}	0.11	–	–	0.69	0.49	0.35	0.41	0.057
MFQ IC₅₀ (nM)	7.1 ± 1.0	12.2 ± 1.1	6.0 ± 1.0	5.9 ± 1.0	6.4 ± 1.2	6.9 ± 0.2	5.8 ± 1.1	6.0 ± 0.72
N	6	6	6	5	5	3	4	4
P vs Dd2 ^{Dd2}	–	0.015	0.48	0.54	0.79	0.55	0.61	0.91
P vs Dd2 ^{Dd2+F145I}	0.48	–	–	0.79	0.79	0.55	0.91	0.35
MFQ IC₉₀ (nM)	21.6 ± 3.2	31.7 ± 3.6	18.5 ± 1.6	20.0 ± 2.5	19.5 ± 2.5	19.8 ± 1.7	16.0 ± 1.5	14.9 ± 2.3
P vs Dd2 ^{Dd2}	–	0.18	0.31	0.79	0.54	0.48	0.35	0.26
P vs Dd2 ^{Dd2+F145I}	0.31	–	–	0.33	0.54	0.48	0.39	0.17

IC₅₀ and IC₉₀ values (nM) are presented as the means ± SEM, as determined in 2 to 7 independent assays performed in duplicate. PPQ, piperaquine; CQ, chloroquine; md-CQ, monodesethyl-chloroquine; md-ADQ, monodesethyl-amodiaquine; QN, quinine; PND, pyronaridine; MFQ, mefloquine; N, number of assays. n.d., not determined. Statistical significance was determined via Mann Whitney U tests. P values are reported for comparisons with the parasite line Dd2^{Dd2} and Dd2^{Dd2+F145I}.

*P < 0.05

**P < 0.01

***P < 0.001

Table S3 Transport in proteoliposomes.

Isoform	PPQ			CQ		
	1-min uptake (pmol/mg)	n	P v. Dd2+F145I	1-min uptake (pmol/mg)	n	P v. Dd2+F145I
Dd2	2.3 ± 0.3	5	0.016	6.5 ± 0.3	6	0.0095
Dd2+F145I	13.6 ± 2.2	4	—	1.9 ± 0.2	4	—
Dd2+F145I+F131C	13.1 ± 2.5	4	>0.99	2.3 ± 0.4	5	0.41
Dd2+F145I+I347T	13.1 ± 2.6	4	>0.99	2.5 ± 0.3	4	0.20
Dd2+F145I+C258W	6.3 ± 0.4	3	0.06	3.9 ± 0.5	3	0.06
3D7	0.5 ± 0.5	5	—	1.2 ± 0.3	4	—

Transport kinetics for ^3H -PPQ and ^3H -CQ were determined with the listed PfCRT variants reconstituted into proteoliposomes. Data (mean ± SD of n = 3-6 experiments) for the 1-min uptake of ^3H -PPQ or ^3H -CQ (depicted in **Fig. 2D and 2E**) are shown for each variant tested.

*P<0.05

**P<0.01

Table S4 All salt bridges found for all PfCRT isoforms.

Amino acid pair	Dd2	Dd2+F145I	Dd2+F145I+F131C	Dd2+F145I+I347T	Dd2+F145I+C258W
ASP57/LYS53	43.58	74.33	60.09	68.34	77.96
GLU54/LYS53	15.88	0.73	0.9	5.79	1.3
GLU54/ARG392	21.6	61.45	29.66	23.2	43.21
GLU54/ARG400	5.96	20.04	7.82	0	1.36
ASP57/LYS56	22.9	3.79	7.16	13.45	2.2
ASP57/ARG400	15.45	0	0.03	3.36	0
GLU207/LYS80	3.06	3.6	12.45	1.5	0
ASP368/ARG81	0	0.3	7.26	5.36	14.95
ASP311/LYS85	45.04	36.15	32.99	64.68	61.09
ASP368/LYS85	5.99	7.52	21.54	3.79	15.41
ASP241/LYS116	17.21	1.2	6.62	0.57	3.7
ASP137/ARG231	66.31	0	0	0	0
GLU208/ARG150	18.64	7.22	26.83	16.74	6.49
GLU198/ARG374	0	0	0	0.9	14.51
GLU204/LYS200	67.64	61.95	36.22	48.17	60.85
GLU204/ARG374	0	20.57	2	0.9	0
GLU208/LYS270	13.52	21.74	19.11	13.68	37.62
GLU232/LYS236	53.73	0	0	0	0
GLU271/LYS270	6.36	11.55	7.39	10.72	10.05
GLU271/LYS307	8.06	4.63	13.52	0.7	0
GLU299/LYS307	14.48	9.39	1.66	15.31	10.59
ASP310/LYS307	13.91	1.76	5.19	0.03	2.43
ASP313/LYS317	25	54.96	0.03	0.4	0
ASP338/LYS339	36.05	0	0	0	0
ASP377/ARG374	55.19	34.25	41.51	69.11	22.34
GLU399/ARG392	60.49	64.08	41.81	57.92	36.09
GLU399/LYS402	1.9	3.53	17.64	2.73	15.01

Interaction lifetimes are given as a percentage of time where the interacting species are within 4 Å of each other. Values shown are for interactions that exist for ≥10% of simulation time for at least one isoform. Green indicates a higher percentage and red indicates a low percentage.

Table S5 (page 1) Averaged log₂fold change of the baseline peptide levels in the variant PfCRT lines versus Dd2^{Dd2+F145I}.

Peptide	Mass Spec Mode	Hb chain	Dd2 ^{Dd2} /	Dd2 ^{Dd2+F145I+F131C} /	Dd2 ^{Dd2+F145I+I347T} /	Dd2 ^{Dd2+F145I+C258W} /
			Dd2 ^{Dd2+F145I}	Dd2 ^{Dd2+F145I}	Dd2 ^{Dd2+F145I}	Dd2 ^{Dd2+F145I}
AHVD	AHVD_pos	Hb α	1.04	0.36	0.47	
AV;LG;GL;VA	AV;LG;GL;VA_pos	Hb β	-5.42	0.44	2.29	-3.46
AVMGN	AVMGN_neg	Hb β	-0.29	-0.05	-0.25	
DALT	DALT_pos	Hb α	-2.48	-0.72	-0.65	
DAVM	DAVM_pos	Hb β				-3.46
DEVGG	DEVGG_pos	Hb β	-10.92	0.78	1.30	-3.39
DGLAH	DGLAH_pos;DGLAH_neg	Hb β				-0.76
DK	DK_pos	Hb α , Hb β				-0.67
DKFLASV	DKFLASV_pos	Hb α				-4.22
DKL	DKL_neg	Hb β	-1.51	-0.79	-2.37	-0.52
DLH;HLD	DLH;HLD_neg	Hb α	-11.85	0.50	1.14	
DLHA;AHLD	DLHA;AHLD_pos	Hb β				-2.77
DLS	DLS_neg	Hb β	-0.91	-0.37	-1.12	-2.98
DLS;LSD;SDL;SLD	DLS;LSD;SDL;SLD_neg; DLS;LSD;SDL;SLD_pos	Hb α				-0.81
	DP_pos	Hb α				-0.54
	DPEN_pos	Hb β	0.54	-0.29	-0.95	-2.16
	DPENF_pos	Hb β				-1.72
	DPVN_pos	Hb α	0.29	-0.63	-1.37	
	DPVNF_pos	Hb α				0.02
	ERM_pos	Hb α				-0.84
	ESFGDLSTP_pos	Hb β	-8.18	0.21	0.25	-1.39
	EV;DL;LD_pos	Hb β				-1.90
	EV;G;GDL;STP;DAV;DGL_pos	Hb β				-1.14
EVGGEA	EVGGEA_pos	Hb β				-3.59
FD	FD_pos	Hb α				-1.39
FLSF	FLSF_neg	Hb α	-0.24	-0.33	-0.49	
GA;AG	GA;AG_neg	Hb α , Hb β				0.21
GAHAGEYGA	GAHAGEYGA_pos	Hb α				0.20
GEALGRLL	GEALGRLL_pos	Hb β	-1.78	1.07	0.72	
GEYG;SFGD;FSDG	GEYG;SFGD;FSDG_pos	Hb α	-12.02	0.93	0.53	-0.59
GKVGKAH;QVKGH	GKVGKAH;QVKGH_pos;GKVGKAH;QVKGH_neg	Hb α				-0.66
GLAH	GLAH_pos	Hb β	-0.07	-0.07	-0.08	
GNPK	GNPK_neg	Hb β	-0.11	-0.65	-0.71	
HFDSHGSQAQ;HVDDMPNALS	HFDSHGSQAQ;HVDDMPNALS_neg	Hb α	-4.40	0.94	1.62	-1.42
	HG;GH: HG_pos	Hb α , Hb β	0.22	-0.54	-0.60	
	HGKKV	HGKKV_neg	Hb α , Hb β			-0.89
	HKLRV	HKLRV_pos	Hb α			-1.91
	HL;LH	HL;LH_pos	Hb β			0.02
	HLD	HLD_neg	Hb β			-2.77
	HLNLKG	HLNLKG_pos	Hb β	-10.94	0.80	0.75
	HVDD	HVDD_pos	Hb α	-4.29	0.28	0.22
	KAHGKK	KAHGKK_pos	Hb β			0.18
	KEFT	KEFT_pos	Hb β			-2.02
KFLASVST	KFLASVST_pos	Hb α	0.29	0.02	0.04	0.37
KGHGK;GHGKK	KGHGK;GHGKK_neg	Hb β	0.96	0.41	0.43	
	KKVADALT;TNVKAAWG;RVDPVNF	KKVADALT;TNVKAAWG;RVDPVNF_pos	Hb α	-2.09	0.80	0.65
	KLRVPDV	KLRVPDV_pos	Hb α			-0.09
	KVNVDDEV	KVNVDDEV_pos	Hb β			-3.34
	LDK	LDK_neg	Hb α			-0.52
	LE	LE_pos	Hb α			-0.19
	LGR;GRL	LGR;GRL_neg	Hb β			0.16
	LH;HL	LH;HL_pos	Hb α	-9.78	0.83	0.49
	LK;KL	LK;KL_pos	Hb β	-0.17	0.58	0.12
	LLGNVLVCVLAH	LLGNVLVCVLAH_neg	Hb β			-2.78
LSPADKTNVKAA	LRVD	LRVD_neg	Hb α	-5.01	1.11	2.28
	LRVPDVN	LRVPDVN_pos	Hb α	0.05	-1.34	-1.24
	LS;VT;SL;TV	LS;VT;SL;TV_pos	Hb α			-0.09
	LSHCLLV	LSHCLLV_pos	Hb α			-2.84
	LSPAD	LSPAD_neg	Hb α	-0.19	-0.21	-0.11
	NP	NP_pos	Hb α			-3.59
	NPKV	NPKV_pos	Hb β	-10.87	0.29	0.40
	NPKVKAHGKK	NPKVKAHGKK_pos	Hb β			-4.09
	NVDE;DEVGG	NVDE;DEVGG_pos	Hb β	-11.27	0.78	0.55
	NVDEVG	NVDEVG_pos	Hb β	-0.07	-0.26	-0.24
NVDEVGGEALG	NVDEVGGEALG_pos	Hb β	0.00	-0.66	-0.35	0.19
	NVKAA;AQVKG;LRVD	NVKAA;AQVKG;LRVD_pos	Hb α	-7.49	0.81	1.63
	PA	PA_pos	Hb α	-7.18	0.77	1.98
	PAD;PDA	PAD;PDA_pos	Hb α	-3.69	-1.51	-1.93
	PAE	PAE_pos	Hb α	-12.24	0.42	0.94
	PD;DDP	PD;DP_pos; PD;DP_neg	Hb β			-0.54
	PDA	PDA_pos	Hb β	-0.11	0.51	-0.04
	PE	PE_pos	Hb β	-1.09	-0.62	-0.79
	PEE	PEE_pos	Hb β			-0.80
	PEEK	PEEK_pos	Hb β			-1.97
PEN	PEN_pos	Hb β				-3.26
	PENF	PENF_pos	Hb β	-6.98	0.93	1.87

Table S5 (page 2) Averaged log₂fold change of the baseline peptide levels in the variant PfCRT lines versus Dd2^{Dd2+F145I}.

Peptide	Mass Spec Mode	Hb chain	Dd2 ^{Dd2} /	Dd2 ^{Dd2+F145I+F131C} /	Dd2 ^{Dd2+F145I+I347T} /	Dd2 ^{Dd2+F145I+C258W} /
			Dd2 ^{Dd2+F145I}	Dd2 ^{Dd2+F145I}	Dd2 ^{Dd2+F145I}	Dd2 ^{Dd2+F145I}
PK	PK_pos	Hb β	-4.73	0.83	1.70	-1.88
PKVK	PKVK_pos	Hb β				-3.52
PN	PN_neg	Hb α	-10.24	0.53	1.48	
PN;NP	PN;NP_pos	Hb α , Hb β	-0.98	-0.62	-0.61	
PNA	PNA_pos	Hb α				-4.42
PNALS	PNALS_pos	Hb α				-2.23
PPVQ	PPVQ_neg	Hb β	-7.96	0.84	1.70	-1.43
PT;TP	PT;TP_pos	Hb α	-8.58	1.00	2.32	-2.88
PTT;DAL	PTT;DAL_pos; PTT;DAL_neg	Hb α , Hb β				-3.71
PV	PV_pos	Hb α	-10.52	0.97	1.76	-2.38
PVN	PVN_pos	Hb α	-3.03	-1.21	-2.54	-4.52
PVNF	PVNF_neg	Hb β	-7.46	1.34	2.28	-5.44
PVQ	PVQ_pos	Hb β				-4.11
PVQA	PVQA_pos	Hb β	-1.13	-0.43	-0.64	-3.96
PWT	PWT_pos	Hb β	0.91	-1.63	-2.14	-2.88
PWTQ	PWTQ_pos	Hb β				-2.84
QKVVA	QKVVA_pos	Hb β	0.15	0.58	-1.27	
RF;FR	RF;FR_pos	Hb β	-2.75	1.30	0.64	
SDLHA;HASLD	SDLHA;HASLD_pos	Hb α				-3.66
SFGD;FSDG	SFGD;FSDG_pos	Hb β	1.97	0.35	0.35	
SFGDLSTP	SFGDLSTP_pos	Hb β				-0.61
SFPPT	SFPPT_pos	Hb α	-0.19	-0.50	0.07	-3.59
STPDAVM;VDPENF	STPDAVM;VDPENF_neg	Hb β				-1.48
TLAA	TLAA_neg	Hb α				-0.52
TNVK	TNVK_pos	Hb α				-0.19
TP	TP_neg	Hb β				-3.44
TPAVH	TPAVH_pos	Hb α	-2.42	1.93	1.02	
TPAVH;KEFT	TPAVH;KEFT_pos	Hb α	0.08	-0.21	-0.30	
TPDA	TPDA_neg	Hb β				-3.30
TPEE	TPE_pos	Hb α				-3.26
TPEEK;LSTPDA	TPEEK;LSTPDA_pos	Hb β				-2.20
TSKY	TSKY_neg	Hb α	0.48	0.30	-0.53	
TYFP	TYFP_pos	Hb α	-1.91	-0.52	-0.76	
V	V_pos	Hb α , Hb β	1.06	-0.06	-0.23	
VAGVANA	VAGVANA_neg	Hb β	0.64	0.46	0.58	
VAGVANAL	VAGVANAL_neg	Hb β	0.47	0.36	0.60	
VAHV	VAHV_pos	Hb α	-9.43	-0.16	0.05	
VAHVDDMP	VAHVDDMP_pos	Hb α	-0.39	0.37	0.11	
VC;CV	VC;CV_neg	Hb β	-9.00	0.70	0.64	-0.42
VD	VD_pos; VD_neg	Hb α , Hb β	-0.17	0.12	-0.27	-1.98
VDD	VDD_neg; VDD_pos	Hb α	-3.43	0.01	1.21	
VDE;DEV	VDE;DEV_pos	Hb β	-2.24	2.00	1.67	-1.03
VDEVG	VDEVG_pos	Hb β				-2.18
VDEVGGEALG	VDEVGGEALG_neg	Hb β	0.72	0.40	0.44	
VPENF	VPENF_pos	Hb β	-0.80	-0.91	-1.61	
VDPVN	VDPVN_neg	Hb α				-2.44
VG	VG_neg	Hb α	-0.18	0.33	0.11	-3.62
VG;GV	VG;GV_neg;VG;GV_pos	Hb β				-3.39
VHAS	VHAS_pos	Hb α	-0.81	-0.68	-1.17	
VHASL	VHASL_neg	Hb α				-3.39
VHL;LHV	VHL;LHV_pos	Hb β				0.30
VLSP	VLSP_pos	Hb α	-1.44	-0.89	-1.87	
VNVDEVG;EVGGEALG	VNVDEVG;EVGGEALG_neg	Hb β				0.17
VT;LS	VT;LS_pos	Hb β	-2.16	0.69	-0.29	
VV	VV_pos	Hb β	-0.04	0.15	-0.07	
VVYP	VVYP_neg	Hb β				-0.79
YH	YH_pos	Hb β				-0.61

134 peptides were detected in either positive or negative mode. Blank spaces denote missing or undetectable values.

Table S6 (page 1) List of peptides showing significantly different levels for Dd2^{Dd2}, Dd2^{Dd2+F145I+F131C}, Dd2^{Dd2+F145I+I347T}, and Dd2^{Dd2+F145I+C258W} compared to Dd2^{Dd2+F145I}.

Peptide	IEP	pH at 5.5	pH at 7.4	P-value	Mean of log ₂ -transformed peak areas of Dd2 ^{Dd2+F145I}	Mean of log ₂ -transformed peak areas of Dd2 ^{Dd2}	Difference in log ₂ -transformed mean peak areas	SE of difference
AHVd	4.78	-0.4	-1	0.048	14.3	15.3	1.0	0.36
AV;LG;GL;VA	5.98; 5.98; 5.98; 5.98	0; 0; 0; 0	0; 0; 0; 0	0.009	11.0	5.6	-5.4	1.05
DGLAH	4.78	-0.4	-1	0.001	12.2	1.3	-10.9	0.68
DLHA;AHLD	4.78; 4.78	-0.4; 0.4	-1; -1	0.002	14.9	3.1	-11.9	0.80
EV;DL;LD	3.64; 3.37; 3.37	-0.9; -1; -1	-1; -1; -1	0.0002	14.7	6.5	-8.2	0.63
GKVGAG;QVKGH	9.37	3.2	2	0.0001	17.1	5.1	-12.0	0.57
HG;GH	7.61	1.7	0	0.003	12.3	7.9	-4.4	0.30
HVDD	3.93	-1.4	-2	0.00002	15.0	4.1	-10.9	0.40
KKVADALT;TNVKAAGW;RVDPVNF	8.76; 9.07; 6.5	1; 1; 0	0.9; 1; 0	0.011	15.0	15.9	15.0	0.96
LH;HL	7.37; 7.37	1.2; 1.2	0; 0	0.00003	12.8	3.1	-9.8	0.47
LRVDPVN	6.5	0	0	0.011	10.6	5.6	-5.0	0.85
NPKVKAHGKK	9.66	4.6	3.9	0.001	11.8	0.9	-10.9	0.78
NVDE;DEVGG	3.29; 3.29	-1.9; -1.9	-2; -2	0.017	14.2	10.4	-3.8	0.86
NVDEVG	3.29	-1.9	-2	0.0001	16.1	4.8	-11.3	0.36
PA	5.98	0	0	0.001	11.7	4.2	-7.5	0.49
PAD;PDA	3.17; 3.17	-2; -2	-2; -2	0.005	10.1	3.0	-7.2	0.93
PD;DP	3.37; 3.37	-2; -2	-2; -2	0.00001	15.1	2.9	-0.1	0.40
PK	9.07	1	1	0.001	12.5	5.5	-7.0	0.76
PKVK	9.37	2	1.9	0.004	11.3	6.6	-4.7	0.76
PN;NP	5.98; 5.98	0; 0	0; 0	0.0004	12.1	1.9	-10.2	0.63
PT;TP	5.98; 5.98	0; 0	0; 0	0.0001	12.9	4.9	-8.0	0.54
PTT;DAL	5.98; 3.37	0; -1	0; -1	0.008	12.2	3.6	-8.6	0.94
PVN	5.98	0	0	0.0001	13.5	3.0	-10.5	0.64
PVNF	5.98	0	0	0.005	14.7	11.7	-3.0	0.54
PVQ	5.98	0	0	0.001	11.0	3.5	-7.5	0.70
VAHVDDMP	3.93	-1.4	-2	0.002	14.0	4.6	-9.4	0.89
VD	3.37	-1	-1	0.005	11.6	2.6	-9.0	0.77

Peptide	IEP	pH at 5.5	pH at 7.4	P-value	Mean of log ₂ -transformed peak areas of Dd2 ^{Dd2+F145I}	Mean of log ₂ -transformed peak areas of Dd2 ^{Dd2+F145I+F131C}	Difference in log ₂ -transformed mean peak areas	SE of difference
NVDE;DEVGG	3.29	-1.9	-2	0.037	14.2	16.0	1.8	0.57

Peptide	IEP	pH at 5.5	pH at 7.4	P-value	Mean of log ₂ -transformed peak areas of Dd2 ^{Dd2+F145I}	Mean of log ₂ -transformed peak areas of Dd2 ^{Dd2+F145I+I347T}	Difference in log ₂ -transformed mean peak areas	SE of difference
DGLAH	4.78	-0.4	-1	0.025	12.2	13.5	1.3	0.34
DLHA;AHLD	4.78; 4.78	-0.4; 0.4	-1; -1	0.029	14.9	16.1	1.1	0.29
HG;GH	7.37; 7.37	0.6; 0.6	0; 0	0.023	12.3	13.9	1.6	0.45
PA	5.98	0	0	0.048	11.7	13.3	1.6	0.47
PAD;PDA	3.17	-2	-2	0.043	10.1	12.1	2.0	0.67
PN;NP	5.98	0	0	0.026	12.1	13.6	1.5	0.35
PT;TP	5.98	0	0	0.022	12.9	14.6	1.7	0.45
PVN	5.98	0	0	0.040	13.5	15.3	1.8	0.55

Table S6 (page 2) List of peptides showing significantly different levels for Dd2^{Dd2}, Dd2^{Dd2+F145I+F131C}, Dd2^{Dd2+F145I+I347T}, and Dd2^{Dd2+F145I+C258W} compared to Dd2^{Dd2+F145I}.

Peptide	IEP	pH at 5.5	pH at 7.4	P-value	Mean of log ₂ -transformed peak areas of Dd2 ^{Dd2+F145I}	Mean of log ₂ -transformed peak areas of Dd2 ^{Dd2+F145I+C258W}	Difference in log ₂ -transformed mean peak areas	SE of difference
AV;LG;GL;VA	5.98; 5.98; 5.98; 5.98	0; 0; 0; 0	0; 0; 0; 0	0.002	13.6	10.2	-3.5	0.51
DEVGG	3.29	-1.9	-2	0.008	14.5	11.1	-3.4	0.61
DKFLASV	6.34	0	0	0.001	15.5	11.3	-4.2	0.35
DLH	4.77	-0.8	-2	0.025	15.8	13.1	-2.8	0.70
DMPNA	3.37	-1	-1	0.031	13.5	12.1	-1.4	0.42
EVGGEA	3.02	-1.8	-2	0.040	12.5	8.9	-3.6	1.00
HLD	4.78	-0.4	-1	0.025	15.8	13.1	-2.8	0.70
KVNVDDEV	4.19	-0.9	-1	0.046	13.7	10.4	-3.3	1.13
LSHCLLV	6.59	0.6	-0.4	0.026	13.9	11.1	-2.8	0.72
LSPAD	3.37	-1	-1	0.013	13.6	10.0	-3.6	0.74
NP	5.98	0	0	0.004	15.3	10.9	-4.4	0.69
NPKV	9.07	1	1	0.028	17.4	13.3	-4.1	0.88
PAE	3.64	-0.9	-1	0.021	14.2	10.8	-3.4	0.81
PEEK	4.44	-0.8	-1	0.034	17.6	15.7	-2.0	0.51
PEN	3.64	-0.9	-1	0.020	15.2	12.0	-3.3	0.77
PENF	3.64	-0.9	-1	0.027	14.7	12.8	-1.9	0.44
PN	5.98	0	0	0.004	15.3	10.9	-4.4	0.69
PTT;DAL	5.98; 3.37	0; -1	0; -1	0.019	12.7	9.0	-3.7	0.85
PVN	5.98	0	0	0.044	16.9	12.4	-4.5	1.18
PVNF	5.98	0	0	0.001	15.4	10.0	-5.4	0.69
PVQ	5.98	0	0	0.012	13.6	9.5	-4.1	0.92
PVQA	5.98	0	0	0.009	15.7	11.8	-4.0	0.77
PWTQ	5.98	0	0	0.026	13.9	11.1	-2.8	0.72
QKVVA	9.07	1	1	0.005	13.3	9.6	-3.7	0.66
TP	5.98	0	0	0.039	14.8	11.3	-3.4	0.92
TPAVH	7.37	0.6	0	0.036	18.1	14.8	-3.3	0.81
TPDAV	3.37	-1	-1	0.013	13.6	10.0	-3.6	0.74
TPE	3.64	-0.9	-1	0.020	15.2	12.0	-3.3	0.77
VG	5.98	0	0	0.002	13.2	9.5	-3.6	0.44
VG;GV	5.98; 5.98	0; 0	0; 0	0.012	14.5	11.1	-3.4	0.46
VHAS	7.37	0.6	0	0.012	14.5	11.1	-3.4	0.46

Data were obtained from three independent experiments with technical triplicates. For each peptide, the isoelectric point (IEP), pH at 5.5, and pH 7.4 are displayed. P values were calculated from unpaired t tests. Gray indicates peptides that were detected in more than one line.

Table S7 List of oligonucleotides used in this study.

Name	Nucleotide Sequence (5'-3')	Description	Lab name	Purpose
p1	CCCTTGTGACCTTAACAGATGGCTC	<i>pfprt</i> exon 2 SalI Fwd	p3519	
p2	TCAAACATGACAAGGGAAATAGT	<i>pfprt</i> exon 5 Rev	p2427	Sequencing primer for <i>pfprt</i> .
p3	CTCGAGatgggtcgctaaactgc	hDHFR Xhol Fwd	p3315	Integration PCR #1. 2.5 kb yes/no.
p4	TTGACCCTTATATATTCCACCA	<i>pfprt</i> 3' UTR	p3403	integration at 3' end Sequences exons 2-3.
p5	cttgggCCCAAGTTGACTGCTTCTAAGC	<i>pfprt</i> 5' UTR (-494-517) Apal Fwd	p3404	Integration PCR #2. 1.2 kb checks integration at 5' end (1.4 kb if unedited due to additional intron 2).
p6	cttatcgatAAGCAGAAGAACATATTAATAG GAATACTTAATTG	<i>pfprt</i> exon 3 ClaI Rev	p3265	
p7	GACCTAACAGATGGCTCAC	<i>pfprt</i> exon 2 EcoRI Fwd	p3264	Integration PCR #3 primer (along with p6). 0.4 kb (0.6 kb if unedited due to additional intron 2). Sequences exons 2-3.
p8	aaccatggatTTATTGTGTAATAATTGAATCGACG	<i>pfprt</i> exon 13 Rev	p1640	Integration PCR #4 primer (along with p5). 2 kb. Sequence entire edited locus.
p9	agccGGTGTGTTGTAAGAGAACCAAGATTATTAG	PfCRT F131C SDM Fwd	p7068	
p10	CTAATAATCTGGTCTcTTACAACATCACCggct	PfCRT F131C SDM Rev	p7069	PfCRT F131C SDM
p11	CCTGTTAGTCATTGGCCatTCATAGGTCTTACA AGAACTAC	PfCRT F145I SDM Fwd	p6106	
p12	GTAGTTCTTGTAAGACCTATGAtGCCAAAATGAC TGAACAGG	PfCRT F145I SDM Rev	p6107	PfCRT F145I SDM
p13	ctttttcaattgttcacttcttgGcttatattac ctgtatacacccctt	PfCRT C258W SDM Fwd	p8550	
p14	aagggtgtatacaggtaatataagCcaagaagtga acaatttgaaaaaq	PfCRT C258W SDM Rev	p8551	PfCRT C258W SDM
p15	aaattttctaccatgacatatactaCtgttagttg tatacaaggtccagca	PfCRT I347T SDM Fwd	p7374	
p16	tgcggacccgtatacacaactaacaGtagtatatg tcatgttagaaaaattt	PfCRT I347T SDM Rev	p7375	PfCRT I347T SDM
p17	GAAGCTTAATTACAATTTCgTGCTATATCCATG TTAGATGCC	PfCRT F131C SDM Fwd	p8136	
p18	GGCATCTAACATGGATATAGCAcAAAATTGTAAT TAAAGCTTC	PfCRT F131C SDM Rev	p8137	F131C SDM on <i>pfprt</i> sequence codon optimized for <i>S. cerevisiae</i> .
p19	GCAGCGTCATCTGGCCatTCATCGGTCTTACCAAGA AC	PfCRT F145I SDM Fwd	p7698	
p20	GTTCTGGTAAGACCGATGAtGCCAAGATGACGCT GC	PfCRT F145I SDM Rev	p7699	F145I SDM on <i>pfprt</i> sequence codon optimized for <i>S. cerevisiae</i> .
p21	GTTAGCTTCTCCAActGTTCACTTCATGgTTAAT CCTGCCAGTTTACACACTACCATTG	PfCRT C258W SDM Fwd	p8821	
p22	GAATGGTAGTGTGAAACTGGCAGGATTAACCATG AAGTGAACAGTTGGAAGAAGCTAAC	PfCRT C258W SDM Rev	p8822	C258W SDM on <i>pfprt</i> sequence codon optimized for <i>S. cerevisiae</i> .
p23	CTCCACCATGACTTACACTAcTGTGAGTTGCATCC AGGGGC	PfCRT I347T SDM Fwd	p8138	
p24	GCCCCTGGATGCAACTCACAgTAGTGTAAAGTCATG GTGGAG	PfCRT I347T SDM Rev	p8139	I347T SDM on <i>pfprt</i> sequence codon optimized for <i>S. cerevisiae</i> .
p25	CCCGCGACTAGTGAGCTCGTCGAC	pFastBac Sequencing	p8157	pFastBac constructs sequencing primer

Fwd, forward primer; Rev, reverse; SDM, site-directed mutagenesis; UTR, untranslated region.

SUPPLEMENTARY REFERENCES

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