Supplementary data:

Mutations in the *Plasmodium falciparum* chloroquine resistance transporter, PfCRT, enlarge the parasite's food vacuole and alter drug sensitivities

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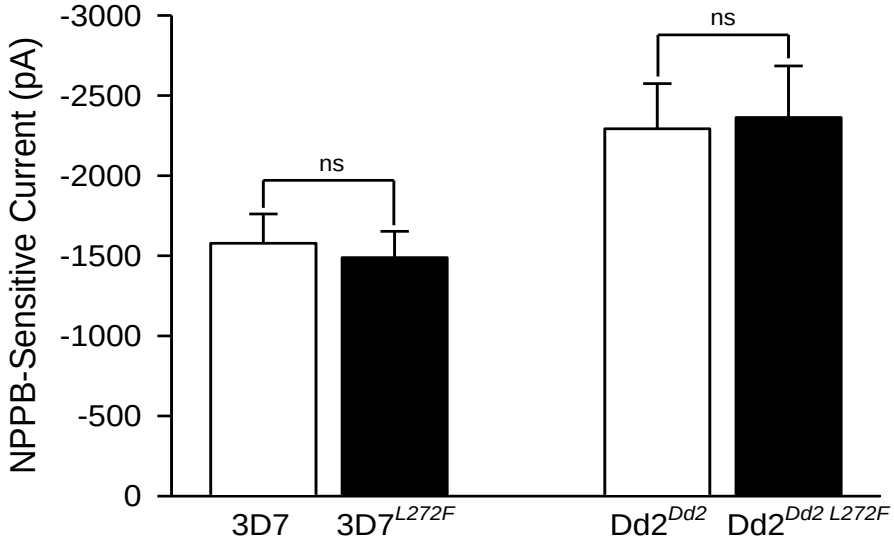
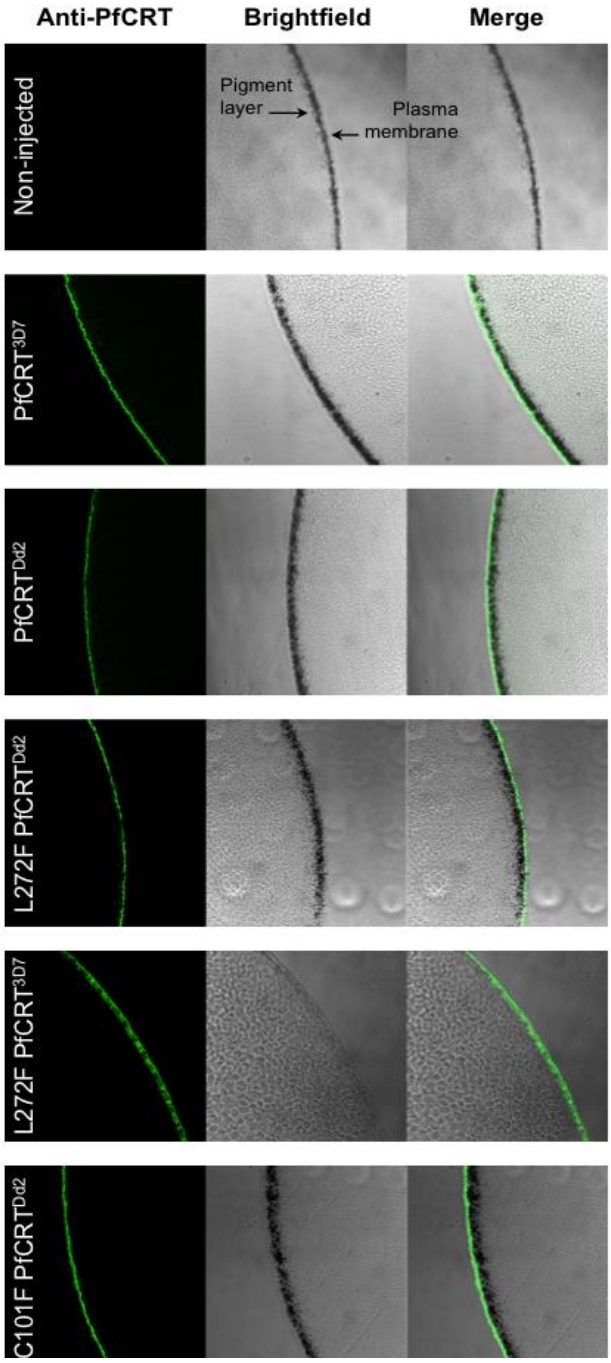


Supplementary Figure S1 | Electrophysiological properties of parasite lines.
Plasmodium parasites are known to alter the permeability of their host's RBC plasma membrane[1]. This altered permeability is characterised by increased transport of a range of structurally unrelated solutes, with a preference for anions over electroneutral and cationic solutes[2]. Using electrophysiological technologies, infected RBCs exhibit greatly increased anionic currents, particularly at negative membrane potentials, that can be inhibited by non-specific anion transport blockers such as 5-nitro-2-(3-phenylpropylamino) benzoic acid (NPPB)[3]**.** BSD pressure has been shown to reduce parasite-induced RBC plasma membrane currents by altering expression of *clag3.1*[4, 5, 6], thus electrophysiological studies were undertaken to compare 3D7 and 3D7^{L272F} and Dd2^{Dd2} and Dd2$^{Dd2\ L272F}$-infected RBCs. The ruptured patch whole-cell voltage-clamp configuration was used to record membrane currents[7]. All experiments were performed at room temperature. Patch pipettes (tip resistances 6 to 12 MΩ) were prepared from borosilicate glass capillaries pulled and polished on a Werner Zeitz DMZ programmable puller. The bath solution contained 155 mM NaCl, 1.4 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, 10 mM glucose (pH 7.4, 310 ± 5 mOsm/kg H_2O). The pipette solution was of the same composition, with the one exception that 1.4 mM $CaCl_2$ was replaced with 0.5 mM EGTA. A 2 ml aliquot of infected RBC suspension (0.001% hematocrit) was placed in a 35 mm Petri dish and left while the RBCs settled. Having obtained a seal (5-15 GΩ) on an infected RBC, the cell was lifted off the bottom of the dish and the patch ruptured by brief application of negative pressure, to attain the whole-cell configuration. Whole-cell currents were recorded using an Axopatch 200B amplifier (digitised at 10 kHz and filtered at 5 kHz with a 4-pole Bessel filter), with voltage command protocols generated and the currents analysed using the pCLAMP software suite (Version 10, Molecular Devices). Whole-cell current/voltage (I-V) curves were obtained by evoking a series of V_T values from -100 to +100 mV in 10 mV steps for 300 ms from a V_H of 0 mV. Current data were measured over the last 50 ms of each record (*i.e.* 250-300 ms). NPPB-sensitive currents at a V_T of -100 mV are shown. NPPB was used at a concentration of 100 µM, which on average inhibited 94 ± 2% of the total current (n = 25). Current data were not included if NPPB inhibition was not greater than 75%, suggesting loss of seal. No significant differences (ns) were found between 3D7 (n = 5) and 3D7^{L272F} (n = 5) and Dd2^{Dd2} (n = 7) and Dd2$^{Dd2\ L272F}$-infected RBCs (n = 8), respectively, suggesting no alteration in the permeability of infected RBCs (Students's *t*-test; p > 0.5).

a



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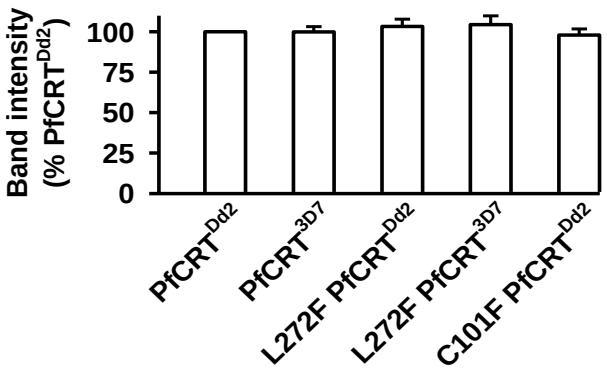
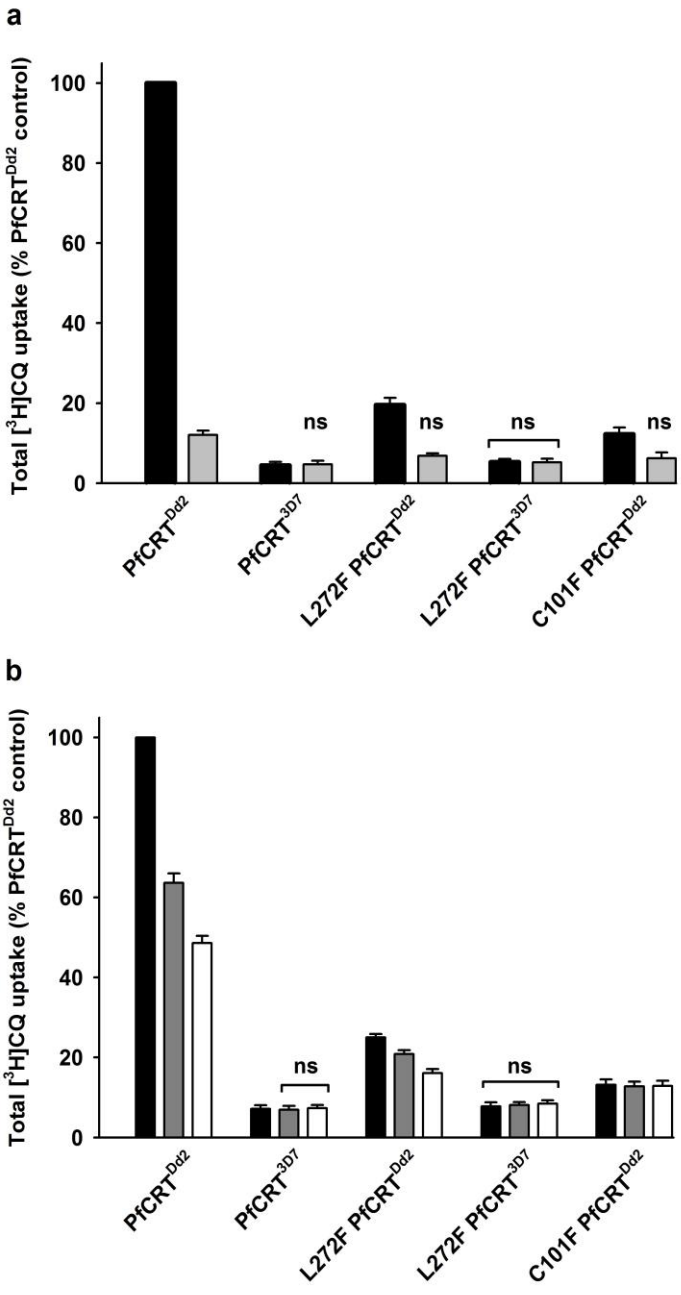


Supplementary Figure S2 | Expression of the C101F and L272F variants of PfCRT in *Xenopus* oocytes. (**a**) Immunofluorescence microscopy was used to localize L272F $PfCRT^{Dd2}$, L272F $PfCRT^{3D7}$, and C101F $PfCRT^{Dd2}$ in the oocyte. In each case, the expression of the PfCRT variant resulted in a fluorescent band external to the pigment layer, indicating that the protein was expressed in the oocyte plasma membrane. The band was not present in non-injected oocytes. (**b**) The level of PfCRT protein in the oocyte membrane was semiquantified using a western blot method[8]. The analysis included $PfCRT^{Dd2}$ as a control, to which the other band intensity values were normalized. Levels of PfCRT protein are shown as mean + SEM from three to five separate experiments (in each experiment measurements were averaged from two independent replicates). There were no significant differences in expression levels between constructs ($p > 0.05$); hence, all of the PfCRT variants were present at similar levels in the oocyte membrane.



Supplementary Figure S3 | Total CQ transport activity of the C101F and L272F variants of PfCRT in *Xenopus* oocytes. (**a**,**b**) The uptake of [^{3}H]CQ into oocytes expressing PfCRT was measured in the absence (closed bars) or presence of 250 µM VP (light grey bars; **a**), 100 µM BSD (dark grey bars; **b**), or 500 µM BSD (open bars; **b**). Within each experiment, measurements were made from 10 oocytes per treatment and uptake was expressed relative to that measured in the PfCRT^{Dd2}-expressing oocytes under control conditions. The normalized data obtained from 4-5 separate experiments (each using oocytes from different frogs) were then averaged and are shown + SEM. These data are total CQ uptake (*i.e.* both CQ accumulation attributable to diffusion and that via PfCRT). In the control treatments, the rates of CQ uptake (pmol/oocyte/h; n = 9 ± SEM) in oocytes expressing PfCRT^{Dd2} and PfCRT^{3D7} were 23.6 ± 2.3 and 1.3 ± 0.2, respectively. 'ns' denotes no significant difference ($p > 0.05$) in CQ accumulation between oocytes expressing a PfCRT variant (in the presence or absence of VP or BSD) and that measured in the PfCRT^{3D7}-expressing oocytes under control conditions.

Supplementary Table S1 | Primers used for sequencing of PfCRT in 3D7 parasites

Name	Sequence (5' to 3')	Direction	Product (bp)
Crt1	CCGTTAATAATAAATACACGCAG	Forward	540
Crt2	GTTCTTGTAAGACCTATGAAGGCC	Reverse	
Crt3	ATCCATGTTAGATGCCTGTTCAGTC	Forward	479
Crt4	CCCAAGAATAAACATGCGAAACC	Reverse	
Crt5	GCTTTTCAAACATGACAAGGG	Forward	581
Crt6	CGACGTTGGTTAATTCTCCTTC	Reverse	
Crt7	GTCTTATATTACCTGTATACACCC	Forward	600
Crt8	CCTTATAAAGTGTAATGCGATAGC	Reverse	

Supplementary Table S2 | PCR verification primers for transfection experiments

Name	Sequence (5' to 3')
p3264	CTTGAATTCGACCTTAACAGATGGCTCAC
p3265	CTTATCGATAAGCAGAAGAACATATTAATAGGAATACTTAATTG
p3315	CTCGAGATGGTTGGTTCGCTAAACTGC
p3403	TTGACCCTTATATATTCCACCCA
p3404	CTTGGGCCCAAGTTGTACTGCTTCTAAGC
p3527	CCTGTATACACCCTTCCATTTTTAAAAGAATTTCATTTACCATATAATGAAATATGGAC
p3528	GTCCATATTTCATTATATGGTAAATGAAATTCTTTTAAAAATGGAAGGGTGTATACAGG

References - Supplementary data

1. Ginsburg, H., Krugliak, M., Eidelman, O. & Cabantchik, Z. I. New permeability pathways induced in membranes of *Plasmodium falciparum*-infected erythrocytes. *Mol Biochem Parasitol* **8**, 177-190 (1983).

2. Kirk, K., Horner, H. A., Elford, B. C., Ellory, J. C. & Newbold, C. I. Transport of diverse substrates into malaria-infected erythrocytes via a pathway showing functional characteristics of a chloride channel. *J Biol Chem* **269**, 3339-3347 (1994).

3. Staines, H. M. *et al.* Electrophysiological studies of malaria parasite-infected erythrocytes: current status. *Int J Parasitol* **37**, 475-482 (2007).

4. Hill, D. A. *et al.* A blasticidin S-resistant *Plasmodium falciparum* mutant with a defective plasmodial surface anion channel. *Proc Natl Acad Sci U S A* **104**, 1063-1068 (2007).

5. Mira-Martinez, S. *et al.* Epigenetic switches in *clag3* genes mediate blasticidin S resistance in malaria parasites. *Cell Microbiol* **15**, 1913-1923 (2013).

6. Sharma, P. *et al.* An epigenetic antimalarial resistance mechanism involving parasite genes linked to nutrient uptake. *J Biol Chem* **288**, 19429-19440 (2013).

7. Staines, H. M. *et al.* Solute transport via the new permeability pathways in *Plasmodium falciparum*-infected human red blood cells is not consistent with a simple single-channel model. *Blood* **108**, 3187-3194 (2006).

8. Summers, R. L. *et al.* Diverse mutational pathways converge on saturable chloroquine transport via the malaria parasite's chloroquine resistance transporter. *Proc Natl Acad Sci U S A* **111**, E1759-1767 (2014).