Modification of *pfap2μ* and *pfubp1* markedly reduces ring-stage susceptibility of *Plasmodium falciparum* to artemisinin *in vitro*

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Abstract

Management of uncomplicated malaria worldwide is threatened by the emergence in Asia of *Plasmodium falciparum* carrying variants of the *pfk13* locus and exhibiting reduced susceptibility to artemisinin. Mutations in two other genes, *ubp1* and *ap2μ* , are associated with artemisinin resistance in rodent malaria and with clinical failure of combination therapy in African malaria patients. Transgenic *P. falciparum* clones, each carrying orthologues of mutations in *pfap2μ* and *pfubp1* associated with artemisinin resistance in *P. chabaudi*, were derived by Cas9 gene editing. Susceptibility to artemisinin and other antimalarial drugs was determined.

Following exposure to 700 nM dihydroartemisinin in the ring-stage survival assay we found strong evidence that transgenic parasites expressing the I592T variant (11% survival), but not the S160N variant (1% survival), of the AP2μ adaptin subunit were significantly less susceptible than were the parental wild-type parasite population. The V3275F variant of UBP1, but not the V3306F variant, also displayed reduced susceptibility to dihydroartemisinin (8.5% survival vs 0.5% survival). AP2μ and UBP1 variants did not elicit reduced susceptibility to 48 hours of artemisinin exposure, or to other antimalarial drugs. Therefore, variants of the AP2 adaptor complex μ-subunit and of the ubiquitin hydrolase UBP1 reduce *in vitro* artemisinin susceptibility at the early ring-stage in *P. falciparum.* These findings confirm the existence of multiple pathways to perturbation of either the mode of action of artemisinin, the parasite's adaptive mechanisms of resistance, or both. The cellular role of UBP1 and AP2μ in *Plasmodium* parasites should now be elucidated.

Introduction

Artemisinin combination therapies (ACT) remain the recommended treatment for uncomplicated *P. falciparum* worldwide.[1] Although ACT are still broadly effective in Sub-Saharan Africa, reduced susceptibility to artemisinin and the partner drug piperaquine has been demonstrated throughout the Greater Mekong subregion, and there is some evidence of decreasing effectiveness in Africa.[2-9] Reduced susceptibility to artemisinin in Southeast Asia has been linked to particular mutations in the propeller domain of the *pfkelch13* (K13) gene. [2,3] However, these mutations are not widely observed in other regions, and the distinct K13 mutations identified at low frequency in Africa do not correlate with either delayed clearance or parasite recrudescence in field studies and travellers. [6,8,9] Reduced artemisinin susceptibility *in vitro* can be generated experimentally by the introduction of variant K13 genes into transgenic parasite lines, [10] but understanding of other genetic determinants of artemisinin and partner drug failure is critical to preserving ACT efficacy worldwide.

In 2007, Hunt *et al* evolved artemisinin-resistant lines of the rodent malaria *P. chabaudi in vivo* and described mutations associated with the phenotype by linkage analysis. [11] These included variants of *pcubp1,* encoding a putative ubiquitin hydrolase, and *pcap2μ,* encoding the μ subunit of the AP2 vesicular trafficking complex. [11,12] Subsequent gene sequencing of peripheral blood parasites from Kenyan patients revealed that the Ser160Asn mutation in the *P. falciparum* homologue of this gene, *pfap2μ,* was associated with ACT treatment failure and that certain alleles of *pfubp1* were also more common among parasites surviving treatment. [13] Similar alleles of *pfubp1* had been previously identified in studies of *in vitro* artemisinin susceptibility of cultured Kenyan isolates of *P. falciparum*.[14] We generated transgenic parasites *in vitro* that harboured an additional, constitutively expressed copy of the modified locus PfAP2μ^{Ser160Asn}, a genotype which currently circulates across Africa.[8,13] Some evidence was found of altered susceptibility to artemisinin and other frontline chemotherapies in these lines, in which the endogenous wild-type locus was also present and active.[15] The availability of genome editing systems in *Plasmodium falciparum* now provides an opportunity to perform gene replacement of the endogenous loci of interest to more directly test the impacts of *pfap2μ* and *pfubp1* mutations on parasite drug susceptibility. [10]

In this study, we used Cas9 editing to generate parasites harbouring the PfAP2μ^{Ile592Thr} orthologue of the PcAP2μ^{Ile568Thr} *P. chabaudi* mutation,[12] and the PfAP2μ^{Ser160Asn} variant seen in Africa.[13] We also introduced into the *pfubp1* locus, separately, sequences encoding the Val3275Phe and Val3306Phe variants, corresponding to *P. chabaudi* mutations previously described by Hunt *et al*.[11] The *in vitro* susceptibility to artemisinin and other frontline antimalarials of these gene-edited parasites was determined and compared to previously validated ART-R *P. falciparum* isolates of Cambodian origin expressing K13 variants.

MATERIALS & METHODS:

Plasmid Design and Construction

Plasmids pL6-AP2µ^{Ile592Thr}-sgDNA and pL6-AP2µ^{Ile592Ile}-sgDNA, including a recodonised donor sequence carrying the mutation of interest, were constructed as described.[16] Constructs to install the *pfap2µ*^{Ser160Asn}, *pfubp1*^{Val3275Phe}, and *pfubp1*^{Val3306Phe} mutations were similarly designed (Supplemental Data).

Parasite Lines

Plasmid constructs were transfected into laboratory clone 3D7. Cambodian origin lines Cam3.II^{R539T}, Cam3.II^{C580Y} and Cam3.II^{REV} were kindly provided by the laboratory of David Fidock. [10] All Cam3.II-lineage parasites harbour wild-type *pfap2µ* and *pfubp1* alleles.

Parasite Culture and Generation of transgenic parasites

Plasmodium falciparum culture was performed in A+ erythrocytes obtained from the UK Blood Bank. Parasites were cultured in RPMI supplemented with Albumax II, L-glutamine, and gentamycin, at 5% haematocrit under 5% CO_2 conditions at 37ºC. Parasites were synchronised using 70% Percoll gradients to capture schizonts and 5% sorbitol to isolate ring-stage parasites.

For transfection, 3D7 parasites were cultured to approximately 10-15% ring-stage parasitaemia at 5% haematocrit under standard conditions. Immediately before transfection, 100 µg of each plasmid was resuspended in 100 µl of sterile TE buffer. 250 µl packed cells equilibrated in 1 x Cytomix (120 mM KCl, 5 mM $MgCl_2$, 25 mM HEPES, 0.15 mM $CaCl_2$, 2 mM EGTA, 10 mM KH_2PO_4/K_2HPO_4, pH 7.6) was combined with 250 µl of 1 x Cytomix in a 2 mm transfection cuvette (Bio-Rad Laboratories) and plasmid DNA was added. The cells were pulsed at 310V, 950 µF, and infinite resistance in a Bio-Rad Gene Pulser, washed and returned to culture. Fresh erythrocytes were added to approximately 5% total haematocrit on day 1 after transfection along with 2.5 nM WR99210 and 1.5 µM DSM-1. Media and selection drugs were replenished every day for 14 days and then every 3 days until parasites were observed by microscopy. Parasites recovered to microscopically detectable levels approximately 3 weeks post-transfection, and genotype was confirmed by PCR-RFLP mapping and Sanger sequencing. In particular, the donor template for installing pf*ap2µ(I592T)* introduced a silent *Spe*I restriction site, and the template for installing *pfap2µ(S160N)* silently ablated an endogenous *Sna*BI restriction site. Both donor templates for *pfubp1* mutations introduced *Hin*dIII restriction sites. Parasites were cloned by limiting dilution, whereby cultures were seeded at 0.3 parasites/well in 250 µl of 2% haematocrit culture in a 96-well plate. Spent media was removed and replaced with complete media supplemented with 0.5% (v/v) fresh red blood cells weekly for three weeks. Parasite growth was examined after three weeks by LDH assay and microscopy, and several clones were selected for genotype confirmation and further characterisation. Approximately one third of each cloning plate contained parasites, consistent with the seeding parasite density.

In vitro *Drug Susceptibility Assays*

Synchronised ring stage parasites at 0.5% parasitaemia and 2% haematocrit were exposed to dihydroartemisinin (DHA), quinine, chloroquine, lumefantrine, piperaquine, and mefloquine over a full 48 h lifecycle in two biological replicates in a 96-well array bearing serial two-fold drug dilutions. The plate was incubated at 37°C for 48 hours and EC_{50} estimates determined as described.[17] $RSA^{0\text{-}4h}$ experiments were carried out exactly as described. [18] FACS was performed on live cells stained with 1:10,000 MitoTracker Deep Red and 1:1,000 SYBR Green (Invitrogen Molecular Probes) in PBS for 20 minutes at 37°C. Cells were stained using 8 volumes of stain solution per volume of culture (2% haematocrit) and analysed using a LSR-II Flow Cytometer (BD Biosciences). Statistical comparisons of drug susceptibility were by the non-parametric Mann Whitney U test.

RESULTS:

AP2μ modifications alter in vitro *susceptibility to frontline chemotherapies*

The orthologue of the *P. chabaudi* AP2μ^{Ile568Thr} variant, [11] PfAP2μ^{Ile592Thr}, was efficiently installed by CRISPR-Cas9 editing onto the *P. falciparum* 3D7 background. In parallel, a PfAP2μ^{Ile592Ile} transgenic line expressing the wild-type Ile592 codon in the context of recodonisation ablating the Cas9 PAM site was also generated (Fig. 1A, 1B; left panels) to rule out a role for silent mutations in drug susceptibility. Both lineages were cloned by limiting dilution, and susceptibility profiles of two clones of each lineage to chloroquine, quinine, DHA, lumefantrine, piperaquine, and mefloquine were characterised. While there was no difference between mutant and wild-type parasites in susceptibility to a 48 h exposure to DHA, quinine, piperaquine, or mefloquine, the 592Thr parasites were more susceptible to lumefantrine (592Thr: 21.3 nM, Ile592: 30.83 nM; mean diff 9.15 nM; P = 0.0067) and chloroquine (592Thr: 8.05 nM, Ile592: 11.88 nM; mean diff 3.83 nM; P < 0.0001) (Table 1). No effect on intra-erythrocytic growth was seen with the Ile592Thr transgenic lines (Suppl. Fig. 1).

We then characterised the susceptibility of these clones to artemisinin using the ring stage survival assay (RSA), [2,18] an *in vitro* correlate of *in vivo* parasite susceptibility in which early ring stage parasites are exposed to a brief pulse of 700 nM DHA. Artemisinin susceptibility was also determined for three Cam3.II-derived Cambodian parasite lines, [10] and the parental 3D7 line, as validated controls. Both clones encoding the PfAP2μ^{Ile592Thr} variant displayed higher ring stage survival rates (clone 1: 11.2%; clone 2: 9.3% survival) than the parental 3D7 strain (1.3% survival). Silent recodonisation had no effect on artemisinin susceptibility, as both gene-edited Ile592Ile clones displayed equivalent artemisinin susceptibility to 3D7 (Fig. 2). For comparison, the Cam3.II Cambodian clinical isolate harbouring the PfK13^{Arg539Thr} variant, and a derived gene-edited line harbouring PfK13^{Cys580Tyr} displayed significantly enhanced survival in the RSA of 28.0% and 17.3%, respectively, when compared to a gene-edited Cam3.II line expressing wild-type K13 (Cam3.II^{REV}: 2.63% survival).

Clones of a lineage expressing PfAP2μ^{Ser160Asn} (Fig. 1A, 1B; right panels), which currently circulates in Africa and was previously associated with delayed parasite clearance in clinical studies, did not show enhanced survival in this assay (Fig. 2). This mutant allele also had no effect on 48 h susceptibility to any of the drugs examined (Table 1). These data confirm that a single amino acid change in AP2μ at codon 592 can confer significantly increased ring-stage survival on an otherwise artemisinin-susceptible genetic background.

The Val3275Phe variant of PfUBP1 *reduces ring-stage artemisinin susceptibility* in vitro

To evaluate the impacts of UBP1 mutation on parasite susceptibility to artemisinin and other frontline chemotherapies, we used Cas9 editing to generate two *P. falciparum* lineages expressing orthologues of the original *P. chabaudi* mutations, encoding PfUBP1^{Val3275Phe} and PfUBP1^{Val3306Phe} (Fig. 3). The

donor templates and sgRNA Cas9 guide sequences used to generate each mutation were identical except for these point mutations. Three weeks after transfection and selection, transgenic parasites were obtained, cloned by limiting dilution, and genotyped by PCR-RFLP and Sanger sequencing.

Two clones of each parasite lineage were examined for their susceptibility to 48 h exposures to dihydroartemisinin, chloroquine, quinine and piperaquine (Table 2). There were no differences in EC_{50} estimates for any chemotherapy between either clone of 3D7-UBP1^{Val3275Phe} and 3D7. Both clones of 3D7-UBP1^{Val3306Phe} were more sensitive than 3D7 to chloroquine. There were no differences in EC_{50} estimates for each drug between the clones of each mutant lineage.

We then examined both clones of each of the *pfubp1* mutant lineages for ring-stage susceptibility to dihydroartemisinin in the RSA (Figure 4). Parasites expressing UBP1^{Val3275Phe} displayed a more than five-fold increase in ring-stage survival compared to wild type 3D7 progenitors (clone 1: 6.46%, clone 2: 6.54%; 3D7: 1.2%; $P < 0.005$). The two Val3306Phe clones displayed equivalent sensitivity to 3D7 (clone 1: 1.46%; clone 2: 1.96%; $P > 0.05$). There was no difference in survival between the two clones within each mutant lineage, and no effect on intra-erythrocytic growth was seen (Suppl. Fig. 1).

DISCUSSION

In this study we present evidence that mutations in genes encoding the μ subunit of the AP2 trafficking complex and the ubiquitin hydrolase UBP1 can generate reduced *P. falciparum* ring-stage artemisinin susceptibility *in vitro* similar to that characteristic of K13-mediated ART-R circulating in the Greater Mekong Subregion. [10] These susceptibility phenotypes are also similar to those seen in the recently described laboratory-evolved parasites of Senegalese origin harbouring PfCoronin variants. [19] Thus, reduced *in vitro* ring-stage susceptibility due to a single amino acid substitution has now been demonstrated for four loci, including *pfk13*.

Resistance to artemisinin has long been proposed to be multigenic. So far, laboratory evolution of reduced artemisinin susceptibility has failed to generate parasites with the most prevalent K13 mutations seen in the field, confirming the importance of examining parasite genetic background when considering drug resistance in the clinic. Resistant variants in K13 only occur naturally in the presence of variants of certain other proteins, perhaps compensating for loss of fitness due to the primary change, [20,21] and this is supported by the results of a recent genetic cross of parasites harbouring *pfk13* C580Y with *pfk13* wild-type parasites. [22] Although *pfk13* genotype at codon 580 determined whether RSA survival was high or low among the progeny, variation within these two groups was modulated by other heritable variant loci. Previous work has implicated intracellular trafficking and protein turnover in the mechanism of artemisinin resistance, [23] and our work here provides further support for these conclusions. In nearly all other systems, the AP2 trafficking complex mediates endocytosis from the plasma membrane, and deubiquitinases are often involved in protein degradation and recycling. A functional characterisation of both proteins is needed to resolve the broader mechanisms underlying this complicated phenotype.

Our findings clarify that the PfAP2μ^{Ser160Asn} mutation has no effect on ring-stage artemisinin susceptibility. Our previous conclusions suggested a possible increase in DHA 48 h-EC_{50} when overexpressing Ser160Asn in a multidrug-resistant background (the Dd2 parasite line from Southeast Asia).[15] However, even though this work was performed before the standardisation of the RSA, we found no evidence of increased survival to a ring-stage artemisinin pulse at that time. Here, using the RSA and 48 h EC_{50} assay formats, we also failed to detect any reduced artemisinin susceptibility mediated by this mutation in a chloroquine-sensitive parasite background (3D7). Further Cas9-editing experiments should be done in the CQ-resistant Dd2 background, but our results to date suggest that Ser160Asn is insufficient to generate reduced artemisinin susceptibility alone, and thus any correlation with ACT failure observed in human infections [8, 13, 15] is due to either other benefits of the genotype, or linkage with other genetic factors. Nevertheless, our RSA results from the Ile592Thr mutant clearly indicate that AP2μ variants can elicit reduced ring-stage parasite susceptibility to artemisinin.

Our data also confirm the ability of variants of UBP1 to mediate reduced artemisinin susceptibility. We examined the UBP1 and AP2µ mutations identified in *P. chabaudi* in isolation to understand their individual contributions to artemisinin sensitivity. In the rodent model, the orthologue of PfUBP1^{Val3306Phe} co-occurred with the orthologue of PfAP2µ^{Ile592Thr}, whereas the latter arose specifically during artemisinin pressure.[11] Consistent with this, in our hands, PfUBP1^{Val3306Phe} is insufficient to generate artemisinin resistance. An obvious next experiment is to examine the effects of these mutations together in a single lineage. Interestingly, neither *pfubp1* mutation conferred any protection from chloroquine, as the rodent model might suggest. However, careful analysis of the linkage data from the seminal experiments of Hunt *et al.* revealed that *pcubp1* mutations were only associated with CQ-R in the context of another mutation in *pcaat1*, an amino acid transporter.[11] As with AP2µ, exploring these combinatorial effects on drug susceptibility in *P. falciparum* will form the basis for important future studies.

The RSA as an *in vitro* test of artemisinin susceptibility phenotype is only a few years old and has been developed in parallel in different labs since it was first described. [24] Inter-lab variability has not as yet been explored with any rigour, but as an initial approach to ensuring comparability between among obtained from different research teams, the inclusion of common control parasite lines is an important step towards standardisation. We were able to use the Cam3.II series of *P. falciparum* lines engineered in the Fidock laboratory as controls in our RSA experiments, and obtained survival estimates for each that were broadly in line with those published in the original work of Straimer *et al.* describing them. [10] On this basis we can confidently compare RSA survival estimates for our transgenic mutant lines back to those obtained for the 3D7 parental line. However, as laboratories differ in the duration of 700 nM DHA pulse used, and on methodology for survival readout (flow cytometry or microscopy), [18,19] further work on assay standardisation is required before inter-lab comparisons of survival estimates will have adequate validity. This is likely to include simple normalisation approaches, based on relative survival compared to agreed standard comparators.

Resistance to artemisinins is unlike that of any other class of antimalarial compounds, being confined to the first few hours of intra-erythrocytic development. We and others have now demonstrated this phenomenon with mutations in *pfk13*, [24] *pfcoronin*, [19] *pfap2µ,* and *pfubp1* as well as with transient pulses of low temperature, [18] suggesting that artemisinin resistance has potentially many genetic and environmental origins. In all these cases so far, trophozoites and schizonts are fully sensitive to artemisinin. Further, even "resistant" ring-stage parasite forms are still mostly sensitive (e.g. more than 70% of parasites are killed by a single 700 nM DHA exposure). It follows that, eventually, even mutant parasite populations should be cleared *in vivo* by extended artemisinin regimens or next-generation analogues with longer half-lives. [25] This pattern also suggests that there is a particular set of cellular processes or circumstances in those first hours of development that are amenable to manipulation by mutation, leading to protection from artemisinin's oxidative mechanism of action. Recent studies and our data here suggest that those processes likely involve intracellular traffic and

protein turnover – two fundamental aspects of parasite biology. Given the success of artemisinins globally, investigations into these mechanisms may reveal other loci capable of manipulating parasite artemisinin sensitivity. Potentially some of these may be of relevance to African settings, where K13 variants have not yet emerged as a cause of treatment failure. Although studies to date in the Mekong region have not described any polymorphisms in the *pfap2mu* locus, this gene has now been identified as lying in a region of chromosome 12 showing evidence of recent strong selection in Ghana & Malawi.[26] Conversely, a mutation in *pfubp1* at codon 3138 was found to be associated with *P. falciparum* isolates from Thai malaria patients carrying the C580Y mutation in *pfk13,* and has thus been implicated in slow clearance after artemisinin therapy.[21] Given that reduced ring-stage artemisinin susceptibility *in vitro* is now confirmed to have multiple potential origins, tracking the phenotypes of delayed parasite clearance and early recrudescence may be more effective than surveillance of specific mutations in African *P. falciparum* populations.

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Author Contributions

RCH conceived, designed, and executed the study. RCH performed cell culture, transfections, and drug susceptibility assays. DAVS planned experiments, performed cell culture and drug susceptibility assays. CJS conceived, designed and supported the study. RCH and CJS wrote the paper, with input from DAVS.

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Competing Financial Interests

The authors declare no competing financial interests.

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Tables

Table 1. Comparison of the *in vitro* susceptibility of *pfap2μ* variant transgenic parasite lines with parental 3D7 exposed to dihydroartemisinin or other antimalarial drugs for 48 h.

Compound	EC_{50} ± SEM (nM)*						
	3D7 parental	AP2μ^{S160N} Clone 1	AP2μ^{S160N} Clone 2	AP2μ^{I591I} Clone 1	AP2μ^{I592I} Clone 2	AP2μ^{I592T} Clone 1	AP2μ^{I592T} Clone 2
Chloroquine	13.7 ± 0.3	13.5 ± 0.1	14.9 ± 0.4	11.4 ± 0.8	12.3 ± 0.4	**8.7 ± 0.9**	**7.4 ± 0.3**
Quinine	31.4 ± 2.6	27.7 ± 2.0	32.3 ± 3.3	23.4 ± 2.5	24.0 ± 2.7	24.8 ± 1.9	23.0 ± 2.3
Piperaquine	31.6 ± 4.5	28.0 ± 3.1	29.6 ± 5.0	23.5 ± 3.6	25.9 ± 0.7	26.5 ± 3.0	22.8 ± 2.5
Mefloquine	12.2 ± 0.2	12.1 ± 0.5	12.0 ± 0.7	16.8 ± 2.4	17.0 ± 1.4	16.2 ± 1.8	16.0 ± 1.9
Lumefantrine	30.0 ± 4.0	26.9 ± 2.7	29.6 ± 3.0	30.2 ± 3.0	31.4 ± 2.3	**20.9 ± 2.1**	**21.8 ± 1.0**
Dihydroartemisinin	3.4 ± 0.3	2.9 ± 0.2	2.7 ± 0.7	2.8 ± 0.6	3.1 ± 0.7	3.1 ± 0.7	2.5 ± 0.4

*EC_{50} values report the mean ± SEM from at least four biological replicates (different cultures, different plates) each performed in technical duplicate. Bold face indicates $P < 0.01$ (Mann-Whitney U test), compared to EC_{50} estimate for 3D7 parental line.

Table 2. Comparison of the *in vitro* susceptibility of *pfubp1* variant transgenic parasite lines with parental 3D7 exposed to dihydroartemisinin or other antimalarial drugs for 48 h.

Compound	EC_{50} ± SEM (nM)*				
	3D7 Parental	Ubp1^{V3306F} clone 1	UBP1^{V3306F} clone 2	UBP1^{V3275F} clone 1	UBP1^{V3275F} clone 2
Chloroquine	18.8 ± 1.4	**13.6 ± 3.0**	**13.7 ± 2.6**	15.3 ± 1.4	14.2 ± 3.0
Quinine	54.9 ± 12	60.5 ± 15	53.8 ± 15	66.8 ± 21	60.6 ± 17
Piperaquine	14.9 ± 6.4	9.8 ± 3.5	8.4 ± 0.9	13.4 ± 2.0	11.0 ± 3.2
Dihydroartemisinin	4.3 ± 0.8	3.3 ± 0.6	3.6 ± 0.6	4.1 ± 0.7	3.8 ± 0.5

*See footnote to Table 1.

Figure Legends

Figure 1. Gene-editing of *pfap2μ*

A CRISPR-Cas9 editing was used to install the *pfap2mu*^{I592T} variant codon (red, left) and *pfap2mu*^{S160N} variant codon (blue, right) into the endogenous locus. The pictured homologous repair constructs were modified to also introduce the I592I wild-type codon in the context of silent mutations ablating the Cas9 PAM site, as a control for their impact on phenotype. Primers used for genotype mapping anneal outside of the homologous repair template as depicted (P1 and P2; Fig. 1, Suppl. Table 1). Recodonised sequence near alternate I592 codons (red) also includes a *Spe*I restriction site, and recodonised sequence near S160 codons (blue) ablates an *SnaBI* restriction site for downstream PCR-RFLP mapping.

B Clones of parasite lines expressing AP2μ^{I592T} and AP2μ^{I592I} (left panel), and AP2μ^{S160N} (right panel) were generated by limiting dilution and confirmed by PCR-RFLP genotype mapping (with *Spe*I or *Sna*BI restriction endonuclease) and Sanger sequencing. Amplification of the locus with P1 and P2 produces a 2247 bp fragment (Suppl. Table 1); for I592I transgenic parasites, *Spe*I digestion of an amplicon containing the transgenic locus liberates a 301 bp fragment. The native amplicon is not cleaved (3D7 lanes). For S160 transgenic parasites, *Sna*BI does not cleave the transgenic amplicon, while wild-type amplicons liberate a 789 bp fragment. MW: fragment length in kilobases.

Figure 2. RSA ^{4h} survival estimates for *pfap2μ* variant transgenic parasite lines (clones 1 and 2) exposed to artemisinin.

RSA$^{4\,h}$ percent survival of mutant and parental parasite lines compared to untreated control, following a 4 h pulse of 700 nM dihydroartemisinin. The Cam3.II-family of parasite lines harbour *pfk13* mutations as indicated; REV indicates wild-type K13 is encoded.[10]. Mean of at least four biological replicates is shown for each line, each performed in technical duplicate, with standard error. Each technical replicate enumerates 100,000 gate-stopping events. P values derived from Mann-Whitney U test, comparing each parasite line to 3D7.

*** P<0.005; NS non-significant;

Figure 3. Gene-editing of *pfubp-1*

A. CRISPR-Cas9 editing was used to install the *pfubp1*^{Val3275Phe} *P. falciparum* orthologue of the previously described *P. chabaudi* mutation into the endogenous locus in 3D7. The same scheme and homology regions were used to install *pfubp1*^{Val3306Phe}. Installation of either mutation introduces a *Hin*dIII restriction site, as marked. Primers P3 and P4 (Suppl. Table 1) were used to perform PCR-RFLP mapping of transgenic clones. B indicates the position of the "Borrmann hotspot" of variation associated with reduced artemisinin susceptibility [13,14]
# indicates position of the Val3306Phe mutation installed using a similarly designed construct.
Coordinates above the gene are in base-pairs.
"in. 2" denotes the position of the second intron in the gene.

B. Clones of parasite lines expressing UBP1^{Val3275Phe} were generated by limiting dilution and confirmed by PCR-RFLP genotype mapping (with primers P3 and P4 and *Hin*dIII restriction digestion) and by Sanger sequencing. Successful integration of the mutant donor template introduces a *Hin*dIII restriction site.

C. Clones of parasite lines expressing UBP1^{Val3306Phe} were generated and confirmed as described for Val3275Phe mutants.

Figure 4. RSA ^{4h} survival estimates for *pfubp1* variant transgenic parasite lines (clones 1 and 2) exposed to artemisinin.

RSA survival of mutant and parental parasite lines compared to untreated control, following a 4 h pulse of 700 nM dihydroartemisinin. Mean of at least four biological replicates is shown for each line, each performed in technical duplicate, with standard error. Each technical replicate enumerates 100,000 gate-stopping events. P values are derived from the Mann-Whitney U test, comparing each parasite line to 3D7.
*** P<0.005; NS non-significant

Figure 1

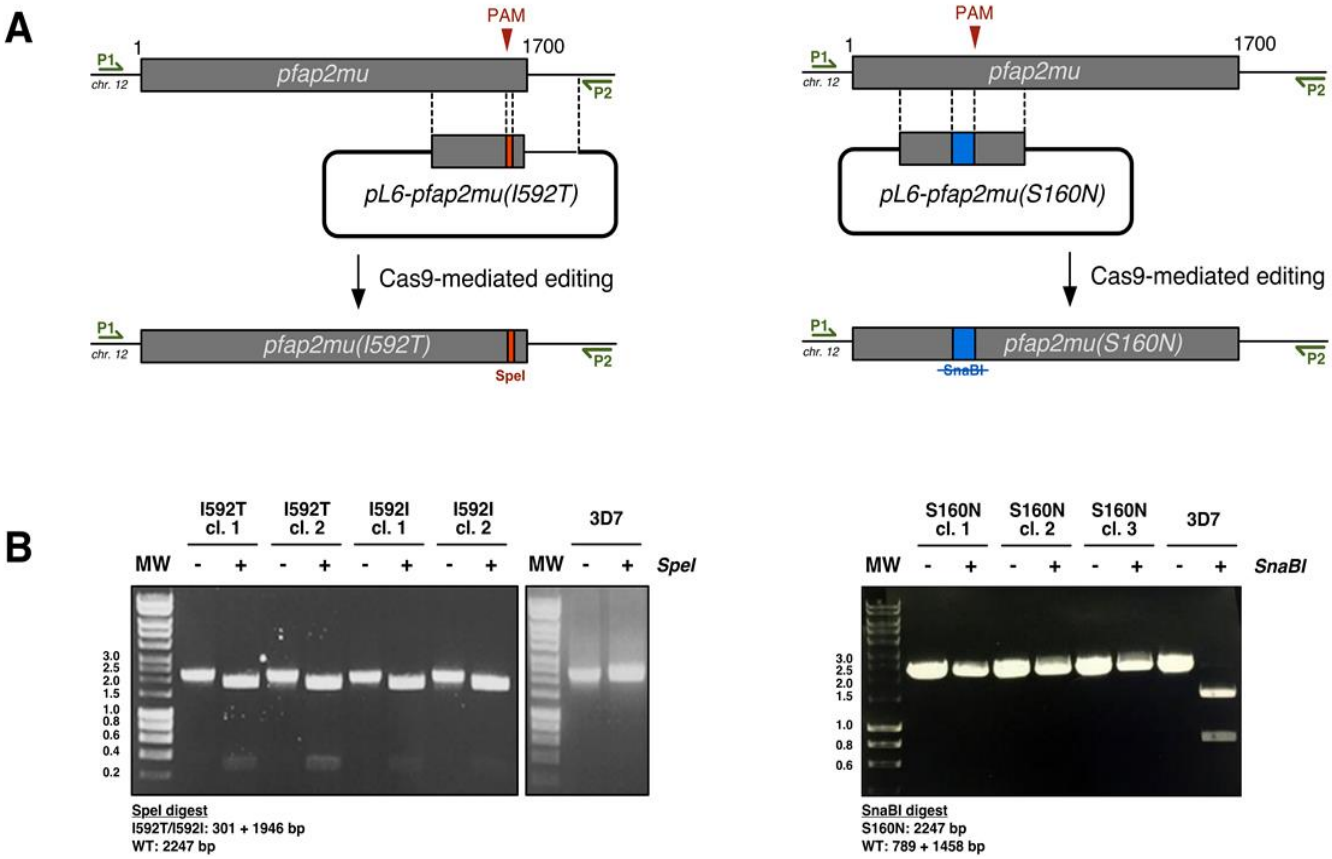

A
PAM
1
1700
P1
chr. 12
pfap2mu
P2
pL6-pfap2mu(I592T)
Cas9-mediated editing
pfap2mu(I592T)
SpeI
pL6-pfap2mu(S160N)
pfap2mu(S160N)
SnaBI
B
I592T cl. 1
I592T cl. 2
I592I cl. 1
I592I cl. 2
3D7
MW
SpeI
S160N cl. 1
S160N cl. 2
S160N cl. 3
SnaBI
3.0
2.5
2.0
1.5
1.0
0.8
0.6
0.4
0.2
SpeI digest
I592T/I592I: 301 + 1946 bp
WT: 2247 bp
SnaBI digest
S160N: 2247 bp
WT: 789 + 1458 bp

Figure 2

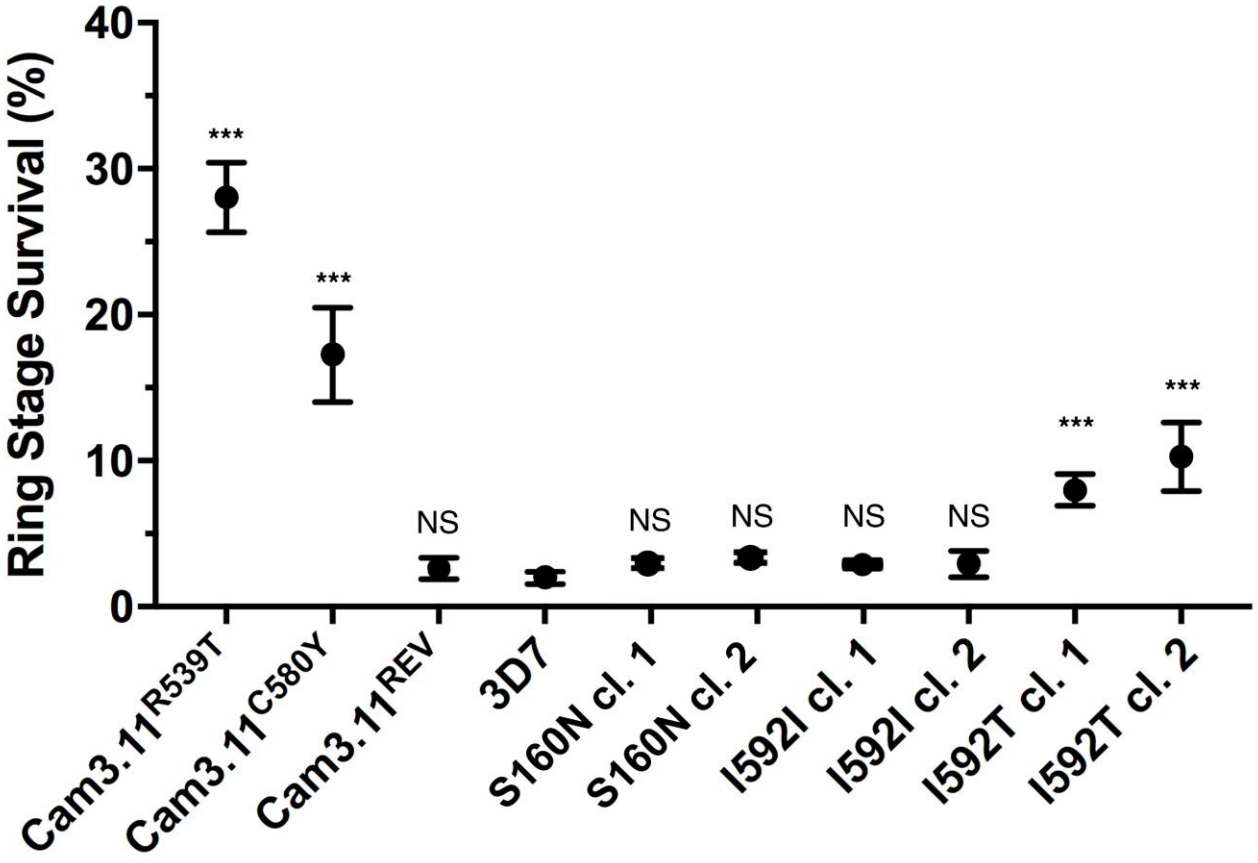

Ring Stage Survival (%)
40
30
20
10
0
***
***
NS
NS
NS
NS
NS
***
***
Cam3.11R539T
Cam3.11C580Y
Cam3.11REV
3D7
S160N cl. 1
S160N cl. 2
I592I cl. 1
I592I cl. 2
I592T cl. 1
I592T cl. 2

Figure 3

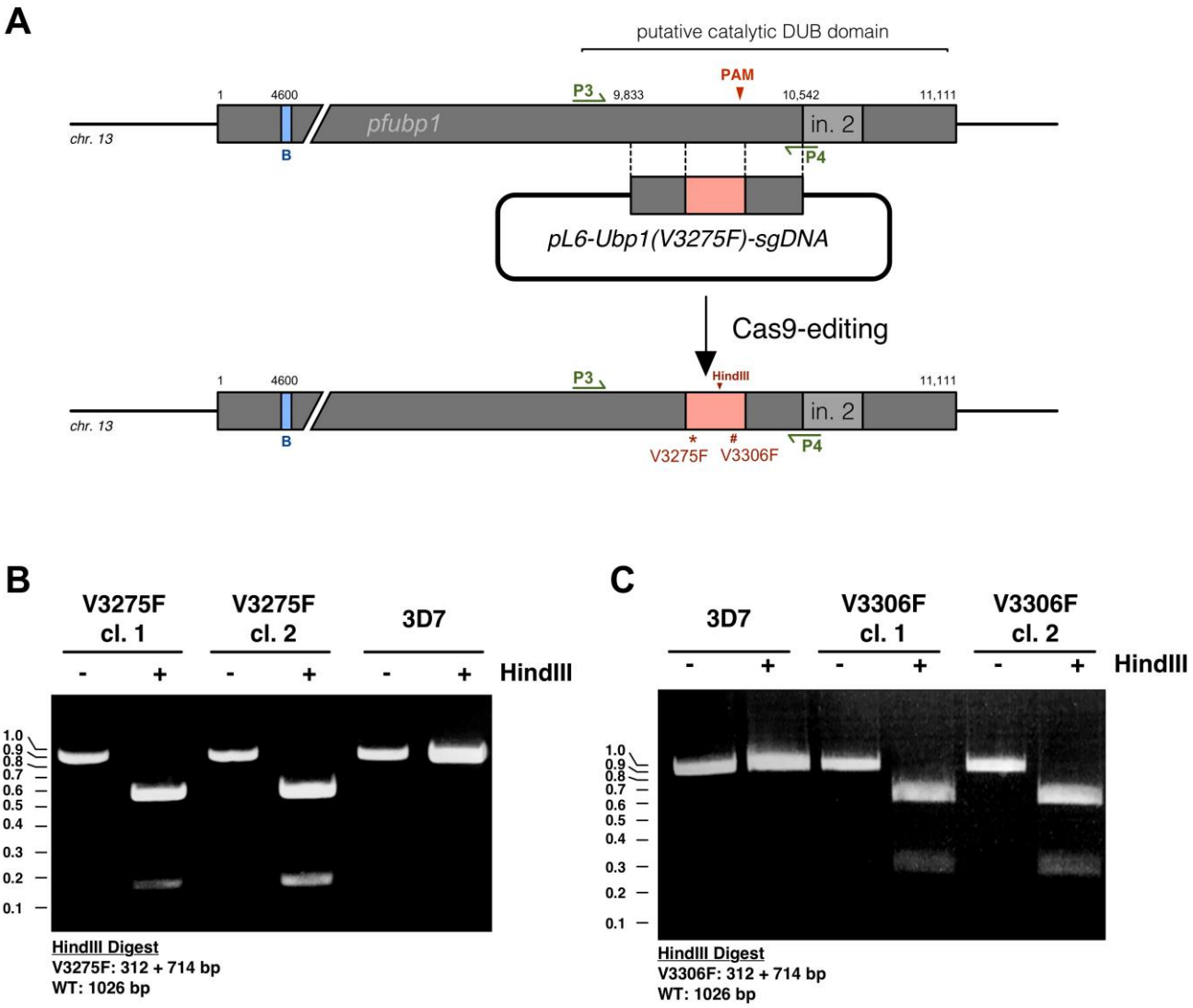
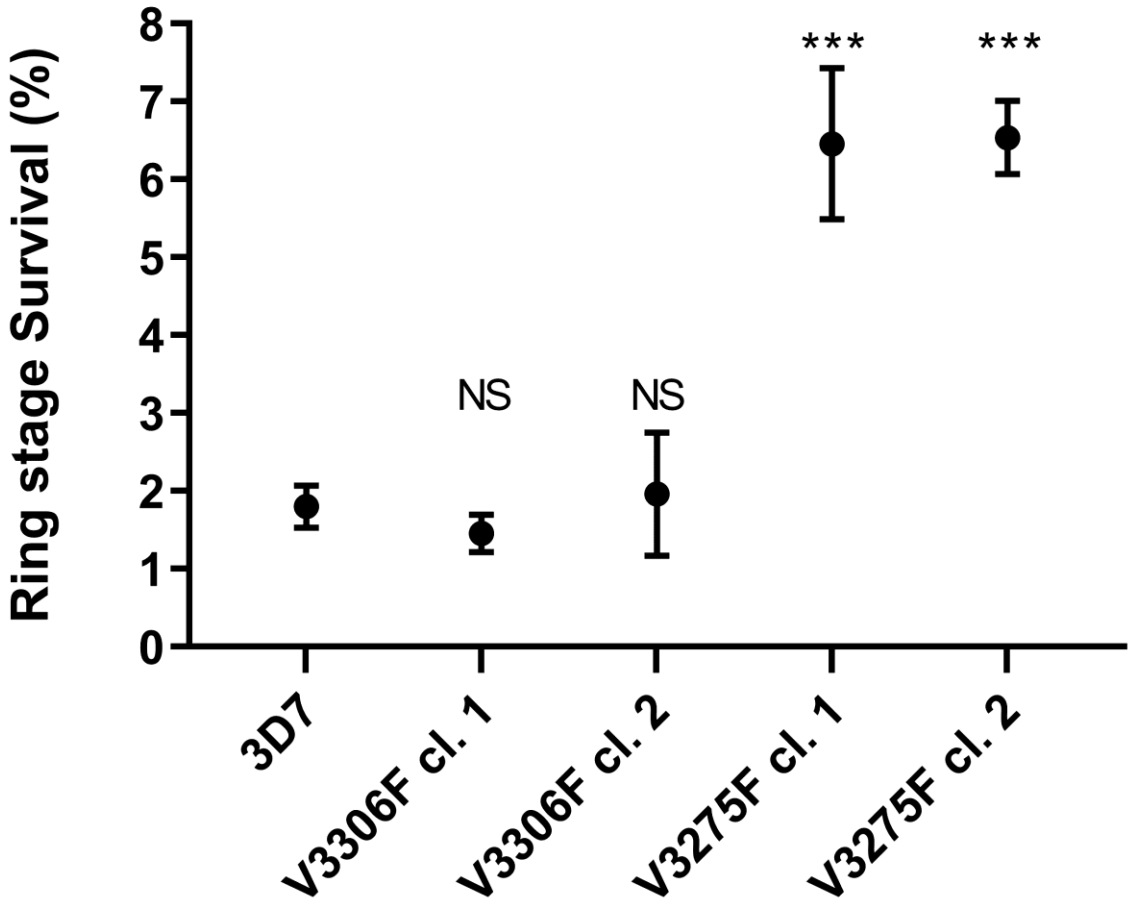

A
putative catalytic DUB domain
PAM
P3
9,833
10,542
1
4600
11,111
pfubp1
in. 2
chr. 13
B
P4
pL6-Ubp1(V3275F)-sgDNA
Cas9-editing
HindIII
V3275F
V3306F
B
V3275F cl. 1
V3275F cl. 2
3D7
HindIII
HindIII Digest
V3275F: 312 + 714 bp
WT: 1026 bp
C
3D7
V3306F cl. 1
V3306F cl. 2
HindIII
HindIII Digest
V3306F: 312 + 714 bp
WT: 1026 bp

Figure 4

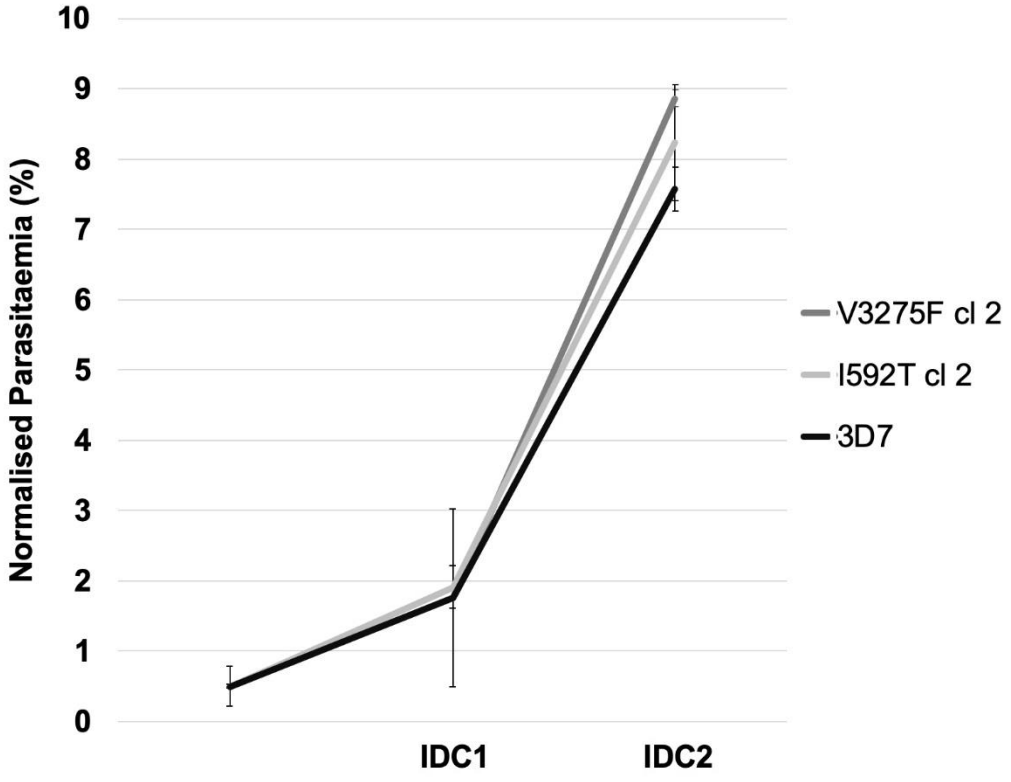

Ring stage Survival (%)
0
1
2
3
4
5
6
7
8
NS
NS
***
***
3D7
V3306F cl. 1
V3306F cl. 2
V3275F cl. 1
V3275F cl. 2

Primer Name	Sequence
P1	5' - AAGACTGTCAAATGTAAAAGACC
P2	5' - CTCATGTAAAACAAAAAGTGAGG
P3	5' - CCATAGGCAAAACTGAATTT
P4	5' - GTCTGATCATAAACATAATATATAATACATAGAAC
P5	5' - TTTTTTTTCTTCCCACATTTCGAA

Suppl. Table 1. Primers used to screen transgenic lines described in this study

Plasmid Name	Purpose
pUF1-Cas9	Express spCas9 with yDHODH selection cassette
pL6-AP2µ(I592T)-sgDNA	Contains *pfap2µ(I592T*) donor template, sgRNA expression cassette for Cas9 editing, and hDHFR selection cassette
pL6-AP2µ(I592I)-sgDNA	Contains *pfap2µ(I592I*) donor template, sgRNA expression cassette for Cas9 editing, and hDHFR selection cassette
pL6-AP2µ(S160N)-sgDNA	Contains *pfap2µ(S160N)* donor template, sgRNA expression cassette for Cas9 editing, and hDHFR selection cassette
pL6-Ubp1(V3275F)-sgDNA	Contains *pfubp1(V3275F*) donor template, sgRNA expression cassette for Cas9 editing, and hDHFR selection cassette
pL6-Ubp1(V3306F)-sgDNA	Contains *pfubp1(V3306F*) donor template, sgRNA expression cassette for Cas9 editing, and hDHFR selection cassette

Suppl. Table 2. Plasmids used to generate transgenic lines described in this study



Supplementary Figure 1. Growth curves for transgenic *P. falciparum* lines

A representative clone of 3D7-Ubp1^{Val3275Phe} and one of 3D7-AP2μ^{Ile592Thr} were grown for 2 successive intra-erythrocytic development cycles (IDC) and parasitaemia measured, compared to the parental 3D7 line. Bars: standard error.