

# Modulation of PF10\_0355 (MSPDBL2) Alters *Plasmodium falciparum* Response to Antimalarial Drugs

Daria Van Tyne,<sup>a</sup> Alessandro D. Ubaldi,<sup>b</sup> Julie Healer,<sup>b</sup> Alan F. Cowman,<sup>b</sup> Dyann F. Wirth<sup>a</sup>

Department of Immunology and Infectious Diseases, Harvard School of Public Health, Boston, Massachusetts, USA<sup>a</sup>; Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia<sup>b</sup>

Malaria's ability to rapidly adapt to new drugs has allowed it to remain one of the most devastating infectious diseases of humans. Understanding and tracking the genetic basis of these adaptations are critical to the success of treatment and intervention strategies. The novel antimalarial resistance locus *PF10\_0355* (*Pfmspdbl2*) was previously associated with the parasite response to halofantrine, and functional validation confirmed that overexpression of this gene lowered parasite sensitivity to both halofantrine and the structurally related antimalarials mefloquine and lumefantrine, predominantly through copy number variation. Here we further characterize the role of *Pfmspdbl2* in mediating the antimalarial drug response of *Plasmodium falciparum*. Knockout of *Pfmspdbl2* increased parasite sensitivity to halofantrine, mefloquine, and lumefantrine but not to unrelated antimalarials, further suggesting that this gene mediates the parasite response to a specific class of antimalarial drugs. A single nucleotide polymorphism encoding a C591S mutation within *Pfmspdbl2* had the strongest association with halofantrine sensitivity and showed a high derived allele frequency among Senegalese parasites. Transgenic parasites expressing the ancestral *Pfmspdbl2* allele were more sensitive to halofantrine and structurally related antimalarials than were parasites expressing the derived allele, revealing an allele-specific effect on drug sensitivity in the absence of copy number effects. Finally, growth competition experiments showed that under drug pressure, parasites expressing the derived allele of *Pfmspdbl2* outcompeted parasites expressing the ancestral allele within a few generations. Together, these experiments demonstrate that modulation of *Pfmspdbl2* affects malaria parasite responses to antimalarial drugs.

Malaria drug resistance poses a serious threat to treatment and control efforts (1, 2). Loci such as *pfprt*, *dhfr*, and *pfmdr1* are all known to play a role in mediating *Plasmodium falciparum* drug resistance and can do so through mutations or copy number variation (CNV) (3), but the precise mechanisms of resistance to many antimalarial drugs are poorly understood. Additionally, these well-known loci do not fully explain the range of responses observed in resistant parasites, suggesting that other loci may be involved in mediating parasite drug sensitivity (4, 5).

*Pfmspdbl2*, also called *PF10\_0355*, *PF3D7\_1036300*, and *MSP3.8*, is a novel antimalarial resistance locus recently identified in a genome-wide association study (GWAS) of 50 global parasite isolates with a high-density single nucleotide polymorphism (SNP) array (6). CNV was also observed at this locus among natural parasite isolates, and parasites with more than one copy of *Pfmspdbl2* tended to be more resistant to halofantrine. Overexpression of either the sensitive or the resistant allele of *Pfmspdbl2* made parasites less sensitive to halofantrine, mefloquine, and lumefantrine but not to structurally unrelated antimalarials. This validated *Pfmspdbl2* as a novel antimalarial resistance locus and confirmed that an increased copy number caused increased resistance to halofantrine and structurally related drugs. However, *Pfmspdbl2* was originally identified through association between SNPs and drug resistance, and the possible effects of these mutations on the modulation of the parasite drug response remained unclear.

MSPDBL2 is a merozoite surface protein containing a Duffy binding-like (DBL) domain, as well as a secreted polymorphic antigen associated with merozoites (SPAM) domain (7). The MSPDBL2 DBL domain binds to red blood cells as a dimer, and binding is dependent on specific metal ions (8). Additionally, the *Pfmspdbl2* gene is highly polymorphic and variably expressed, ap-

pears to be under balancing selection, and is a likely target of host immunity (9, 10). Immunofluorescence studies have shown that MSPDBL2 is located on the merozoite surface in both schizonts and free merozoites (7, 8), despite the lack of a membrane anchor.

Here we further characterize the role of *Pfmspdbl2* in mediating parasite responses to antimalarial drugs. We wanted to probe the differential effects of CNV and SNPs on drug sensitivity, and we did this through knockout and allelic replacement experiments. We found that in addition to CNV, SNPs within *Pfmspdbl2* also affect parasite drug responses. Additionally, we found large difference in long-term fitness when parasites expressing different *Pfmspdbl2* alleles competed directly under drug pressure.

## MATERIALS AND METHODS

**Parasite culture, drug testing, and knockout parasite lines.** Parasites were maintained under standard culture conditions (11) in RPMI medium supplemented with 5% human O<sup>+</sup> serum and 5% Albumax II. Where indicated, parasites were synchronized with 5% D-sorbitol. Drug testing of knockout and allelic replacement lines was performed by measuring parasite incorporation of tritium-labeled hypoxanthine (12). Drug assays were run with at least two technical replicates of each data point, and 50% inhibitory concentration (IC<sub>50</sub>) data were averaged over multi-

Received 21 December 2012 Returned for modification 15 February 2013

Accepted 28 March 2013

Published ahead of print 15 April 2013

Address correspondence to Dyann Wirth, [dfwirth@hsph.harvard.edu](mailto:dfwirth@hsph.harvard.edu).

Supplemental material for this article may be found at <http://dx.doi.org/10.1128/AAC.02574-12>.

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doi:10.1128/AAC.02574-12

ple assays (either duplicate or triplicate), as referenced in the figure legends. Drug data from Senegalese parasites (see Fig. 2 for a summary) were generated with a high-throughput SYBR green I-based assay and have been previously reported (13). In all cases,  $IC_{50}$ s were calculated with GraphPad Prism (GraphPad, San Diego, CA) by using a four-parameter, log-logistic nonlinear regression of fluorescence intensity versus  $\log_{10}$ -transformed drug concentrations.

*Pfmspdbl2* knockout parasites were generated by double-crossover recombination in the 3D7 parasite background with the pCC1 vector (14) (A. D. Uboldi et al., unpublished data). See Fig. S1 in the supplemental material for the experimental design and validation of locus disruption.

**Whole-genome sequencing and the association between *Pfmspdbl2* SNPs and halofantrine sensitivity.** Whole-genome sequencing of 45 culture-adapted parasite lines from Senegal was conducted, and genome-wide association studies were performed previously (13). The SNP Pf\_10\_001435509 (by PlasmoDB v5.0 coordinates; also known as Pf\_10\_001434265 in PlasmoDB v5.5-v7.2 and Pf\_10\_001434268 in PlasmoDB v9.1) encodes a cysteine-to-serine point mutation at position 591 within the SPAM domain of MSPDBL2. SNP calls at this position in 3D7, Dd2, and the *Plasmodium reichenowi* Oscar strain were verified previously (6).

**Allelic replacement parasites.** Allelic replacement vectors were generated by cloning the *Pfmspdbl2* gene sequence minus the first 74 nucleotides from Dd2 (derived [DR] allele) and Senegal P26.04 (ancestral [AC] allele) parasites into the pHH1 vector (15). Plasmids were transfected into the Dd2 parasite line, and stable transfectants were selected with 2.5 nM WR99210 (16). Transfectants were twice cycled off WR99210 for 2 weeks each time, and stable integration and loss of episome were confirmed by Southern blotting with ClaI, NotI, and a probe generated with the following primers: F, 5'-GGG GAA AGC ATA TAA TAA TAC TAT AGA TGC-3'; R, 5'-CTT GGA GGA ACA AGA ACC CCC TTA TTA TCA-3'. Protein expression of hemagglutinin (HA)-tagged MSPDBL2 in the Dd2 DR line was measured by Western blotting with anti-HA and anti-lactate dehydrogenase (LDH) monoclonal antibodies with the LI-COR system (LI-COR Biosciences, Lincoln, NE).

Immunofluorescence assays were performed by harvesting mixed-stage parasites growing in culture, pelleting the red blood cells, and washing them with  $1 \times$  phosphate-buffered saline (PBS) before fixing them in a rotating suspension with 4% paraformaldehyde–0.0075% glutaraldehyde for 30 min at room temperature. After washing, parasites were permeabilized in a rotating suspension with 0.1% Triton X-100 and 3% bovine serum albumin in  $1 \times$  PBS for 45 min at room temperature. Parasites were then blocked in 3% bovine serum albumin in  $1 \times$  PBS for at least 1 h, followed by rat anti-HA 3F10 antibody (150 ng/ml) staining overnight at 4°C. After washing, parasites were stained with Alexa 488-conjugated anti-rat antibodies (1:750 dilution) for 1 h at room temperature. Cells were mounted with Vectashield mounting medium with 4',6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Burlingame, CA) and imaged with the  $100\times$  objective on a Nikon Eclipse TE300 microscope. Images were obtained with MetaMorph software v7.5 (Sunnyvale, CA) with a Hamamatsu C4742-95 camera and processed with Adobe Photoshop CS 5.0 (Adobe, San Jose, CA).

**Growth competition experiments.** Competition experiments were performed by mixing synchronized ring stage cultures of DR and AC parasite lines in equal ratios either in the absence of drug or in the presence of 1 nM halofantrine. Parasites were harvested after 1, 4, 8, or 14 asexual cycles, and genomic DNA was extracted. Ratios of each line were determined by quantitative real-time PCR with a custom TaqMan assay designed to detect the two different alleles of SNP Pf\_10\_001435509. The sequences of the assay primers used were as follows: F, 5'-GGG TCA TCA TCT CTT GAA CAA CAC T-3'; R, 5'-TCG CTT TCA TTA GCT ATC TGT TCA ATA TCC-3'. The sequences of the probes used were as follows: Allele 1 (AC; VIC), 5'-CAA TTC TAA AGC ACT TCC CTT-3'; Allele 2 (DR; 6-carboxyfluorescein, FAM), 5'-ATT CTA AAG CAC ATC CCT

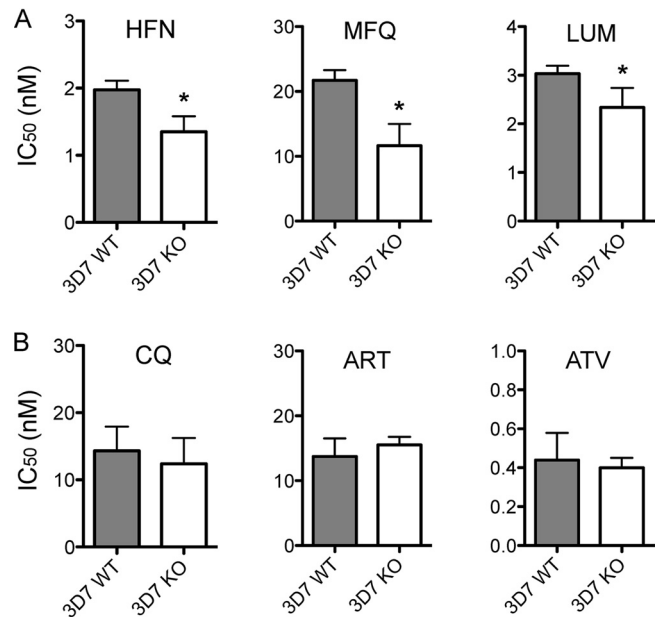


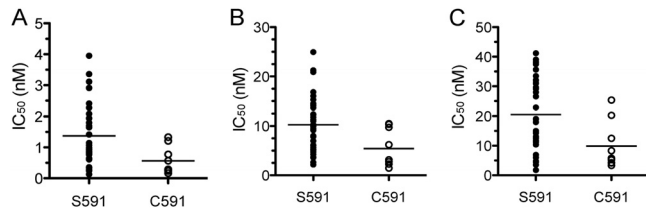
FIG 1 (A) Drug responses of 3D7 wild-type (WT) and *Pfmspdbl2* knockout (KO) lines to halofantrine (HFN), mefloquine (MFQ), and lumefantrine (LUM), measured by tritium-labeled hypoxanthine incorporation. (B) Drug responses of 3D7 WT and KO lines to chloroquine (CQ), artemisinin (ART), and atovaquone (ATV). The values shown are means  $\pm$  standard deviations of at least two separate assays run on different days. \*,  $P < 0.05$  (two-tailed, unpaired  $t$  test).

T-3'. Cycle threshold values were normalized to a standard curve of known genomic DNA mixtures of the two parasite lines.

## RESULTS

**Loss of *Pfmspdbl2* makes parasites more sensitive to halofantrine, mefloquine, and lumefantrine.** The *Pfmspdbl2* locus was successfully disrupted in the 3D7 parasite line (Uboldi et al., unpublished data). See Fig. S1 in the supplemental material for design and validation of locus disruption. Drug testing by tritium-labeled hypoxanthine incorporation showed that parasites in which *Pfmspdbl2* had been disrupted were more sensitive than 3D7 wild-type parasites to halofantrine, mefloquine, and lumefantrine (Fig. 1A). Parasites lacking a functional *Pfmspdbl2* gene were unchanged in their responses to chloroquine, artemisinin, and atovaquone (Fig. 1B).

**A mutation within the SPAM domain of *Pfmspdbl2* is associated with parasite responses to halofantrine, mefloquine, and lumefantrine.** SNP 1434268 (in PlasmoDB v9.1; 1434265 in PlasmoDB v5.5-v7.2 and 1435509 in PlasmoDB v5.0) on chromosome 10 encodes a nonsynonymous cysteine-to-serine mutation at position 591 within the SPAM domain of *Pfmspdbl2*. Both the *P. reichenowi* Oscar strain and the *P. falciparum* 3D7 line have cysteine at position 591 (6), suggesting that cysteine is the ancestral (AC) allele and serine is the derived (DR) allele at this position. We previously tested 45 culture-adapted parasites that were recently isolated from infected patients in Senegal with a SYBR green I-based drug assay (13). Only 9 of the 45 Senegalese parasites tested have cysteine at this position and 36 have serine, resulting in a DR allele frequency of 80% in this population. Parasites with C591 were more sensitive to halofantrine, mefloquine, and lumefantrine than those with S591 were (Fig. 2,  $P < 0.05$  for each drug).



**FIG 2** A mutation at position 591 within the SPAM domain of *Pfmspdbl2* is associated with parasite responses to halofantrine (A), mefloquine (B), and lumefantrine (C) in 45 Senegalese parasites. SNP calls and drug data are from reference 13; horizontal lines show the mean  $IC_{50}$ s of the groups.  $P = 0.021$  for halofantrine,  $P = 0.016$  for mefloquine, and  $P = 0.014$  for lumefantrine (two-tailed unpaired  $t$  tests of S591 versus C591).

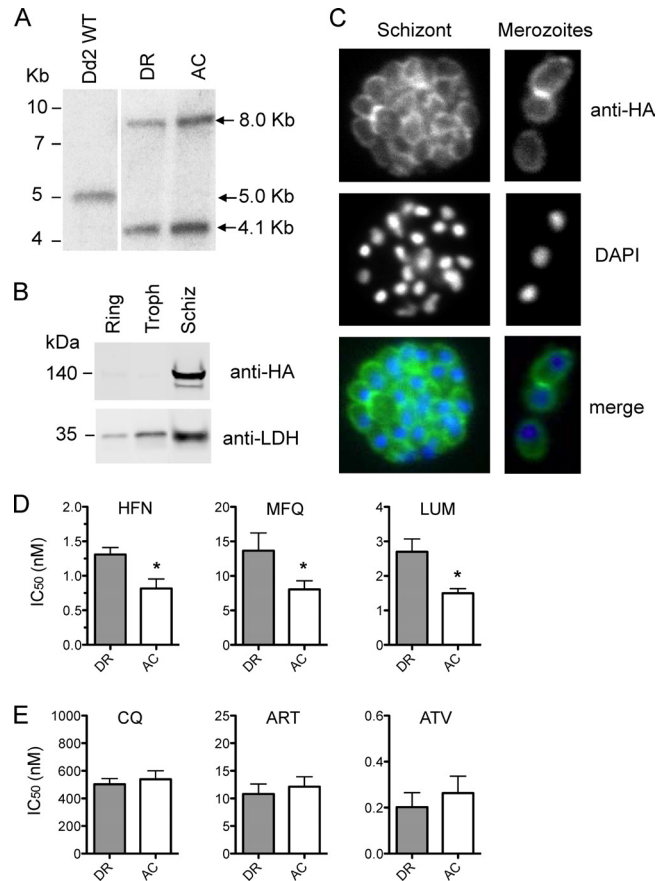
C591S was not significantly associated with parasite sensitivity to chloroquine, artemisinin, or atovaquone (data not shown).

To further investigate the association between the C591S mutation and parasite drug responses, we generated Dd2 parasites where a 3' portion of the endogenous *Pfmspdbl2* locus was replaced with a triple-HA-tagged version of either the DR or the AC allele of this gene. Stable integrants containing the DR or the AC construct were confirmed by Southern blotting (Fig. 3A). Western blotting of lysates from the DR line with anti-HA antibodies showed expression of the tagged protein in schizonts and the presence of two bands of around 140 kDa (Fig. 3B). The expected size of HA-tagged MSPDBL2 is 90 kDa; however, mass spectrometry analysis confirmed that the two 140-kDa bands identified both corresponded to MSPDBL2 (data not shown). Immunofluorescence assay showed the proper localization of the HA-tagged protein on the merozoite surface (Fig. 3C). Drug testing by incorporation of tritium-labeled hypoxanthine revealed that AC parasites were more sensitive to halofantrine, mefloquine, and lumefantrine than DR parasites were (Fig. 3D) but showed similar responses to chloroquine, artemisinin, and atovaquone (Fig. 3E).

**Parasites expressing the *Pfmspdbl2* DR allele outcompete parasites expressing the AC allele under drug pressure.** We were interested in determining if the observed differences between DR and AC drug responses affect the fitness of parasites when they are cultured over many cycles. DR and AC parasite lines were synchronized, and ring stage parasites were mixed in a 1:1 ratio. Parasite mixtures were allowed to grow in normal medium containing no drug or in medium containing 1 nM halofantrine, and the ratio of DR to AC parasites was determined with a TaqMan SNP assay after 1, 4, 8, and 14 asexual replication cycles (Fig. 4). The ratio of the two parasite lines was largely unchanged in the absence of drug pressure, remaining close to the 1:1 starting ratio. However, when parasites were incubated with 1 nM halofantrine, DR parasites quickly outcompeted AC parasites, reaching an almost 5-fold excess after eight replication cycles. These results show that the *Pfmspdbl2* DR allele has no associated fitness cost in the absence of drug pressure and furthermore suggest that modest  $IC_{50}$  differences may have large effects on parasite fitness when parasites compete under drug pressure.

## DISCUSSION

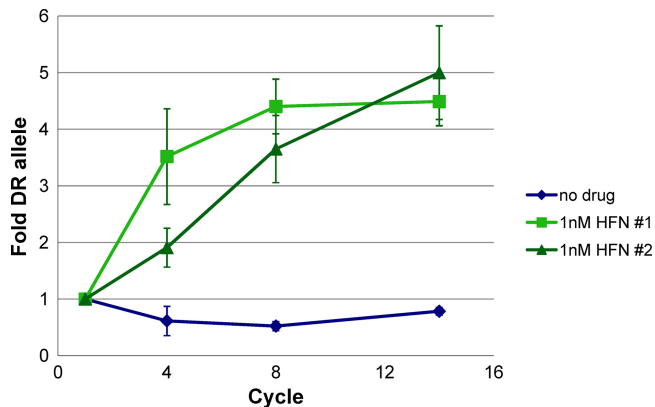
Drug resistance poses a serious threat to efforts aimed at eliminating malaria from regions where it is endemic, and considerable attention is currently focused on understanding the molecular basis of resistance. Nonetheless, precise mechanisms of antimalar-



**FIG 3** *Pfmspdbl2* mutations in the absence of CNV affect parasite responses to halofantrine and structurally related antimalarials. (A) Southern blot assays of Dd2 wild-type (WT) and *Pfmspdbl2* allelic replacement parasites where the endogenous genome locus has been replaced with HA-tagged versions of the DR allele (S591 cloned from Dd2) or the AC allele (C591 cloned from Senegal P26.04). Digestion of the wild-type, untagged *Pfmspdbl2* locus with ClaI and NotI generates a single band of 5.0 kb, while digestion of the HA-tagged integrated locus generates two bands of 4.1 and 8.0 kb. (B) Western blotting of ring, trophozoite (Troph), and schizont (Schiz) stage cultures of the DR line with anti-HA and anti-LDH antibodies shows expression of HA-tagged MSPDBL2 in schizonts. (C) Immunofluorescence of a representative schizont and representative merozoites of the DR line with anti-HA antibodies (green) and DAPI staining (blue). (D) Drug responses of DR and AC lines to halofantrine (HFN), mefloquine (MFQ), and lumefantrine (LUM) measured by tritium-labeled hypoxanthine incorporation. (E) Drug responses of DR and AC lines to chloroquine (CQ), artemisinin (ART), and atovaquone (ATV). The values shown are means  $\pm$  standard deviations of at least three separate assays run on different days. \*,  $P < 0.05$  (two-tailed unpaired  $t$  test).

ial drug action and drug resistance remain poorly defined for many antimalarials (17). Transgenic parasite lines, created and tested under controlled and standardized laboratory conditions, allow the precise study of how subtle genetic changes can contribute to drug resistance. Even if the effects are modest, elucidation of the impacts of individual loci on drug responses can deepen our understanding of how parasites may eventually become clinically resistant.

We wanted to know how CNVs and SNPs within *Pfmspdbl2* alter parasite susceptibility to antimalarials, in order to better understand the role of this locus in mediating *P. falciparum* drug responses. Previous work showed that an increased *Pfmspdbl2* copy number made parasites more resistant to halofantrine and



**FIG 4** *Pfmspdbl2* DR allele parasites outcompete AC allele parasites in the presence of halofantrine. Parasites were mixed at equal ratios and grown with no drug or 1 nM halofantrine (HFN). Ratios of DR and AC parasites were determined after 1, 4, 8, and 14 asexual cycles by quantitative real-time PCR with TaqMan probes and are shown as fold abundance of the DR allele. Two biological replicates (#1 and #2) are shown, and error bars show standard deviations of technical replicates.

structurally related antimalarials (6). Consistent with this result, we found that parasites lacking expression of *Pfmspdbl2* were more sensitive to halofantrine, mefloquine, and lumefantrine but not to structurally unrelated drugs. In the absence of *Pfmspdbl2* expression, parasites showed no growth defects compared to wild-type parasites, suggesting that this gene is not essential for asexual parasite growth but its absence makes parasites more sensitive to a specific class of antimalarials. These findings further support a role for *Pfmspdbl2* in modulating parasite drug responses, as decreased drug sensitivity seems to scale with gene copy number in a manner similar to that of CNVs at other drug resistance loci such as *pfmdr1* and *pfgh1* (18, 19).

We focused on a specific mutation (C591S) within MSPDBL2 for two reasons. First, in the original global GWAS that identified *Pfmspdbl2* as a novel antimalarial resistance locus, the genome-wide significant association signal mapped to the 3' portion of the gene and spanned the C591S mutation (6). Second, C591S was the mutation within *Pfmspdbl2* that was most strongly associated with parasite sensitivity to halofantrine in a recent whole-genome sequence-based GWAS (13), although this association did not reach genome-wide significance. Importantly, only 20% of the 45 sequenced Senegalese parasites in that study retained the AC allele at position 591. This suggests that selection of some sort, such as the use of halofantrine or perhaps more likely the structurally related antimalarials mefloquine and lumefantrine, has driven the DR allele to high prevalence in this population.

Other studies have documented cases where drug resistance-associated mutations confer a fitness disadvantage in the absence of drug pressure (20, 21), but this does not seem to be the case with *Pfmspdbl2*. This could be due to the modest change in drug response conferred by the C591S mutation or perhaps because compensatory mutations that restore parasite fitness have occurred. Because we used genomic DNA from natural parasite isolates to generate the allelic replacement constructs used here, the AC parasite line contains additional mutations within *Pfmspdbl2* besides C591S. While it remains possible that mutations other than or in addition to C591S could underlie the changes in drug response that we observed, these results confirm that mutations within

*Pfmspdbl2*, in the absence of CNV, also mediate parasite drug responses.

The precise mechanism by which MSPDBL2 affects antimalarial drug responses remains unknown. MSPDBL2 localizes to the merozoite surface, despite lacking a membrane anchor. Other merozoite surface proteins are known to form complexes (22), and it seems possible that MSPDBL2 could act in concert with other surface proteins. Additionally, we wondered whether MSPDBL2 binds to halofantrine directly; however, attempts to investigate this were confounded by high rates of nonspecific drug binding to a reference protein (data not shown). The C591S mutation resides within the SPAM domain of MSPDBL2; the SPAM motif has yet to be characterized and could have a function different from that of the DBL domain, which appears to mediate binding to erythrocytes (8). Alternately, perhaps parasites are better able to bind and invade red blood cells in the presence of certain antimalarials when they have more copies of *Pfmspdbl2* or when the protein is in a specific conformation.

Overall, these experiments demonstrate that genetic manipulation of *Pfmspdbl2* affects drug sensitivity of *P. falciparum*. Similar to other malaria parasite drug resistance genes, we find effects of both CNVs and SNPs in modulating parasite drug responses. Importantly, our findings demonstrate that large differences in parasite fitness can be revealed when parasites are grown over multiple generations under drug pressure, as they likely would in the natural setting of infected patients.

#### ACKNOWLEDGMENTS

We thank Bradley Coleman, Manoj Duraisingh, Jeffrey Dvorin, and Ulf Ribacke for providing reagents, helping design experiments and interpret results, and helpful discussion. We also thank Daniel Park and Pardis Sabeti for help analyzing whole-genome sequencing data.

This work was supported by the Bill and Melinda Gates Foundation.

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