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K13-propeller mutations confer artemisinin resistance in *Plasmodium falciparum* clinical isolates

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The emergence of artemisinin resistance in Southeast Asia imperils efforts to reduce the global malaria burden. We genetically modified the *Plasmodium falciparum K13* locus using zinc-finger nucleases, and measured ring-stage survival rates following drug exposure in vitro; these rates correlate with parasite clearance half-lives in artemisinin-treated patients. With isolates from Cambodia, where resistance first emerged, survival rates decreased from 13–49% to 0.3–2.4% following the removal of K13 mutations. Conversely, survival rates in wild-type parasites increased from ≤0.6% to 2–29% following the insertion of K13 mutations. These mutations conferred elevated resistance to recent Cambodian isolates compared to reference lines, suggesting a contemporary contribution of additional genetic factors. Our data provide a conclusive rationale for worldwide K13-propeller sequencing to identify and eliminate artemisinin-resistant parasites.

The worldwide use of artemisinin (ART)-based combination therapies (ACTs) for the treatment of *Plasmodium falciparum* malaria is the foundation of renewed efforts to eradicate this leading cause of childhood mortality (*1*, *2*). The pharmacodynamic properties of clinically-used ART derivatives (artesunate, artemether and dihydroartemisinin (DHA)) can reduce the biomass of drug-sensitive parasites by 10^4-fold every 48 hours (*3*), corresponding to a single cycle of asexual blood-stage *P. falciparum* development. The short half-life (typically <1 hour) of ART derivatives in plasma necessitates the use of longer-lasting partner drugs that can eliminate residual parasites once the ART component has dropped to sub-therapeutic concentrations (*4*). The use of ACTs in expanded malaria control and elimination programs has yielded notable successes in recent years, contributing to an estimated 30% reduction in global mortality rates in the past decade (*5*).

These impressive gains, however, are now threatened by the emergence of ART resistance, first detected in western Cambodia and now observed in Thailand, Vietnam and Myanmar (*6*, *7*). The severity of this situation is underscored by the fact that resistance to piperaquine, an ACT partner drug, is emerging in western Cambodia (*8*, *9*). No alternative, fully effective first-line therapy is currently available to replace ACTs, should ART fail globally. Clinically, ART resistance is defined as a long parasite clearance half-life (the time it takes for the peripheral blood parasite density to decrease by 50%) following treatment with ART monotherapy or an ACT (*6*, *10*, *11*). This metric correlates with the percentage of early "ring-stage" parasites (0–3 hours post-invasion of human erythrocytes) that survive a pharmacologically relevant exposure to DHA (the active metabolite of ARTs), as measured in the in vitro Ring-stage Survival Assay ($RSA_{0\text{-}3h}$) (*12*).

Recently, mutations in the propeller domain of the *K13* gene were identified as candidate molecular markers of ART resistance (*13*). This gene resides on chromosome 13 of the *P. falciparum* genome, near regions earlier associated with slow parasite clearance rates (*14–16*). K13 belongs to the kelch superfamily of proteins whose propeller domain harbors multiple protein-protein interaction sites and mediates diverse cellular functions, including ubiquitin-regulated protein degradation and oxidative stress responses (*17*). The K13 M476I mutation was first observed in Tanzanian F32 parasites that were exposed in vitro to escalating concentrations of ART over 5 years (yielding the F32-ART line (*13*, *18*)). Subsequent genomic analysis of Cambodian isolates

identified four prevalent K13-propeller mutations (Y493H, R539T, I543T and C580Y) that were associated with elevated $RSA_{0\text{-}3h}$ survival rates in vitro and long parasite clearance half-lives (>5 hours) in patients (*13*, *19*). Determining whether K13-propeller mutations confer ART resistance in clinical isolates and assessing the contributions of individual polymorphisms in distinct genetic backgrounds is essential to defining the underlying molecular mechanisms.

We developed zinc-finger nucleases (ZFNs (*20*)) to enable targeted genetic engineering of *K13* in newly culture-adapted Cambodian isolates and older established reference lines of *P. falciparum* (tables S1, S2). ZFNs were introduced into cultured intra-erythrocytic parasites via electroporation with plasmids containing *K13* donor templates. ZFNs triggered double-stranded breaks in the *K13* genomic target locus of this haploid organism, leading to DNA resection and repair events that captured mutations delivered by $pZFN^{K13}$-h*dhfr* plasmids (fig. S1). Donor plasmids contained additional synonymous mutations that preclude ZFN binding while preserving the K13 translated amino acid sequence across that same stretch of DNA base pairs. These silent ZFN binding-site mutations thereby protected the donor sequence and prevented the edited recombinant locus from being re-cleaved by the nucleases. Plasmids contained either the wild-type *K13* allele or one of several mutations (present in the six-blade K13-propeller domain) found in ART-resistant Cambodian isolates or F32-ART. This strategy successfully introduced or removed mutations in a set of *P. falciparum* clinical isolates from Cambodia, the epicenter of emerging ART resistance, as well as reference laboratory lines from distinct geographic origins (Fig. 1; table S3). Of note, $RSA_{0\text{-}3h}$ assays comparing parental and edited control parasites showed no difference if only the binding-site mutations were introduced into the K13-propeller domain, indicating that these synonymous mutations were phenotypically silent (Fig. 1; fig. S2). Independent assays with the same parasite lines tested by our different groups yielded consistent survival rates between laboratories (fig. S3).

Using donor plasmids containing a wild-type *K13*-propeller sequence and silent binding-site mutations, we generated a series of clones in which individual K13 mutations were removed from ART-resistant Cambodian isolates. One of these isolates (Cam3.II) showed slow clearance following ART monotherapy (in vivo half-life 6.0 hours; table S2). Parental $Cam3.I^{R539T}$ and $Cam3.II^{R539T}$ isolates harboring the R539T mutation showed 40–49% $RSA_{0\text{-}3h}$ survival, while edited $Cam3.I^{rev}$ and $Cam3.II^{rev}$ clones carrying the reverted wild-type allele showed only 0.3–0.7% survival (Fig. 2A, B; table S4). These highly significant differences in the survival rates of ring-stage parasites exposed to elevated DHA concentrations confirm the importance of R539T in mediating in vitro ART resistance in Cambodian isolates. Significant reductions in $RSA_{0\text{-}3h}$ survival rates were also observed upon removal of I543T (43% in $Cam5^{I543T}$ versus 0.3% in $Cam5^{rev}$; Fig. 2C) and C580Y (13% in $Cam2^{C580Y}$ versus 2.4% in $Cam2^{rev}$; Fig. 2D).

We also assessed the impact of introducing K13 mutations into a fast-clearing Cambodian isolate (CamWT; in vivo half-life 3.7 hours; table S2), the $Cam3.II^{rev}$ clone, and three reference lines (V1/S, F32-TEM and FCB). CamWT and $Cam3.II^{rev}$ parasites harboring wild-type *K13* alleles showed 0.6–0.7% $RSA_{0\text{-}3h}$ survival, while the corresponding C580Y-edited clones yielded 9% and 24% survival, respectively (Fig. 2E, F). Introducing R539T into V1/S caused a similar increase in $RSA_{0\text{-}3h}$ survival (0.3% to 21%; Fig. 2G; table S4). Editing F32-TEM to express M476I caused a moderate increase in $RSA_{0\text{-}3h}$ survival (<0.2% in F32-TEM to 1.7% in $F32\text{-}TEM^{M476I}$; Fig. 2H). We also observed modest in vitro resistance in FCB parasites edited to express C580Y, with $RSA_{0\text{-}3h}$ survival increasing from 0.3% in the parental line to 1.9% in FCB^{C580Y} parasites (Fig. 2I). This result differs from a recent study of the use of CRISPR in *P. falciparum*, which reported a greater increase in $RSA_{0\text{-}3h}$ survival (11–15%) in two clones engineered to express K13 C580Y (*21*). That report used the drug-sensitive NF54 strain, isolated decades before ART use and the emergence of resistance (*22*), and did not examine additional mutations or assess the impact of removing K13 mutations from ART-resistant clinical isolates.

In contrast to the significant changes we observed in the $RSA_{0\text{-}3h}$, standard in vitro dose-response measurements using parental and *K13*-edited V1/S and Cam3.II parasites revealed no effect of R539T or C580Y on DHA or artesunate IC_{50} values (fig. S4). This finding is consistent with earlier studies that showed no correlation between IC_{50} values and clinical ART resistance (*6*, *10*, *12*).

We subsequently investigated whether individual mutations confer different levels of ART resistance in the $RSA_{0\text{-}3h}$. In the Dd2 reference line, the introduction of M476I, R539T or I543T mutations conferred considerably higher degrees of resistance than Y493H and C580Y (10–30% versus 2-4% survival, respectively; Fig. 2J; table S4). These data corroborate the recent observation of higher levels of in vitro resistance in Cambodian isolates containing the R539T mutation as compared to Y493H or C580Y (*23*).

The relatively modest increase in survival of C580Y-expressing Dd2 parasites compared to R539T- and I543T-expressing clinical isolates and edited clones was quite unexpected, given that C580Y has rapidly become the predominant mutant allele in western Cambodia (*7*, *13*). We thus explored the impact of C580Y in different genetic backgrounds. Introducing C580Y conferred greater levels of resistance in three Cambodian isolates as compared to Dd2 and FCB parasites (Fig. 2K), suggesting a role for additional parasite factors in augmenting K13-mediated resistance in these contemporary field isolates. The disparity between relatively low in vitro resistance conferred by C580Y and its widespread dissemination in Cambodia might be explained by a lower fitness cost or increased transmission potential of

C580Y-expressing parasites, or by the parasite genetic background.

Cambodian parasites are uniquely characterized by sympatric subpopulations that show only limited genetic admixture and that generally harbor distinct K13 mutations (*16*). These findings suggest that K13 mutations might have arisen preferentially on backgrounds with favorable genetic factors. In this context, recent comprehensive analyses of K13 mutations across multiple sites in Southeast Asia have documented a series of additional mutations associated with slow clearance rates in Cambodia, Thailand, Myanmar, Laos and Vietnam (*7*, *24*). K13 mutations have also been observed in African isolates (*7*, *25*, *26*), although none of these correspond to the most prevalent mutations in Cambodia, and ART or ACT treatments in African sites continue to show a high level of efficacy (*7*). Of note, a recent deep-sequencing study of the K13-propeller domain in over 1,110 *P. falciparum* infections collected from 14 sites across sub-Saharan Africa identified a large reservoir of naturally-occurring K13-propeller variation, whose impact on artemisinin susceptibility is unknown and requires further investigation. These polymorphisms include one rare mutation previously observed in Cambodia (P553L), and several others (including A578S) close to known resistance-causing mutations in the propeller domain (26). Our gene-editing system can now be used to comprehensively dissect K13 polymorphisms across malaria-endemic regions and identify those that confer ring-stage ART resistance.

Mode-of-action studies have shown that ARTs are active against all asexual blood stages of parasite development. In the more mature trophozoite stages, ARTs are activated following hemoglobin degradation and liberation of reactive heme whose iron moiety can cleave the endoperoxide linkage of these sesquiterpene lactone drugs (*27*). Activation generates free radicals that are thought to trigger oxidative stress and damage cellular macromolecules including parasite membrane components, proteins and neutral lipids (*28*, *29*). Recent evidence suggests that hemoglobin degradation begins early after merozoite invasion, potentially providing a source of ART activator in ring-stage parasites (*30*). Our $RSA_{0\text{-}3h}$ data support earlier evidence that reduced ring-stage susceptibility accounts for the clinical phenotype of slow parasite clearance following ART treatment (*12*, *31*). K13 mutations might achieve this by protecting parasites from the lethal effects of ART-induced oxidative damage, potentially via a cellular pathway similar to antioxidant transcriptional responses regulated by the mammalian ortholog Keap1 (*32*). Our set of K13-modified isogenic parasites with different levels of ART resistance on distinct genetic backgrounds now enables a search for K13-interacting partners and delivers tools to interrogate the underlying mechanism.

Our data demonstrate a central, causal role for K13-propeller mutations in conferring ART resistance in vitro, and provide a molecular explanation for slow parasite clearance rates in patients (*6*, *7*, *10*). By exposing greater parasite biomasses to ACTs in vivo, K13-propeller mutations may promote the evolution of partner drug resistance (*8*, *9*) and higher-grade ART resistance. Our study thus offers a conclusive rationale for a global *K13* sequencing effort to track the spread of ART resistance and mitigate its impact on malaria treatment and control programs, particularly in hyperendemic regions in Africa.

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respectively, filed by Institut Pasteur. These patents cover the use of K13 mutations as a molecular marker of *P. falciparum* ART resistance. Sangamo BioSciences, Inc. holds patents on engineered DNA-binding proteins and the use thereof in targeted genome engineering and gene-specific regulation. All other authors declare no competing financial interests.

SUPPLEMENTARY MATERIALS

www.sciencemag.org/cgi/content/full/science.1260867/DC1
Materials and Methods
Figs. S1 to S4
Tables S1 to S5
Statistical data
References (*33*–*36*)

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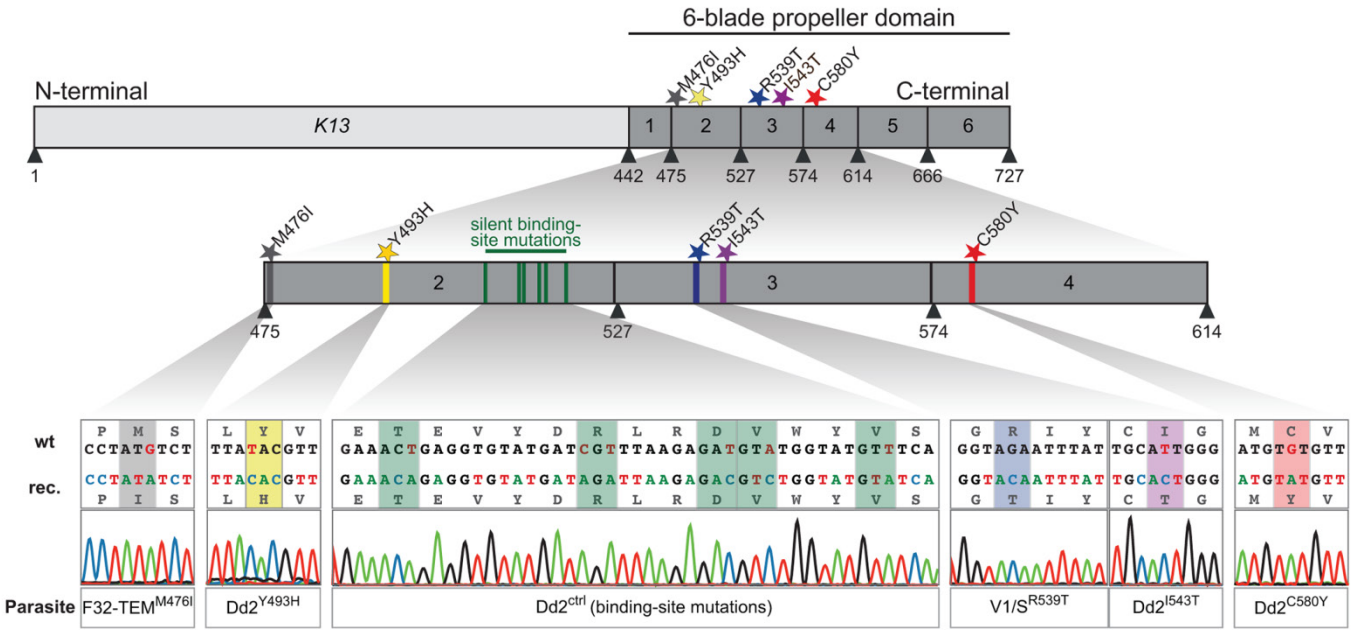

Fig. 1. Genetic modification of the K13-propeller domain. Location of K13-propeller mutations, and sequencing results showing the insertion of individual mutations into recombinant parasites used in the Ring-stage Survival Assay ($RSA_{0\text{-}3h}$). $Dd2^{ctrl}$ parasites contain only silent binding-site mutations and showed 0.7% survival rates, equivalent to those of parental Dd2 parasites (fig. S2).

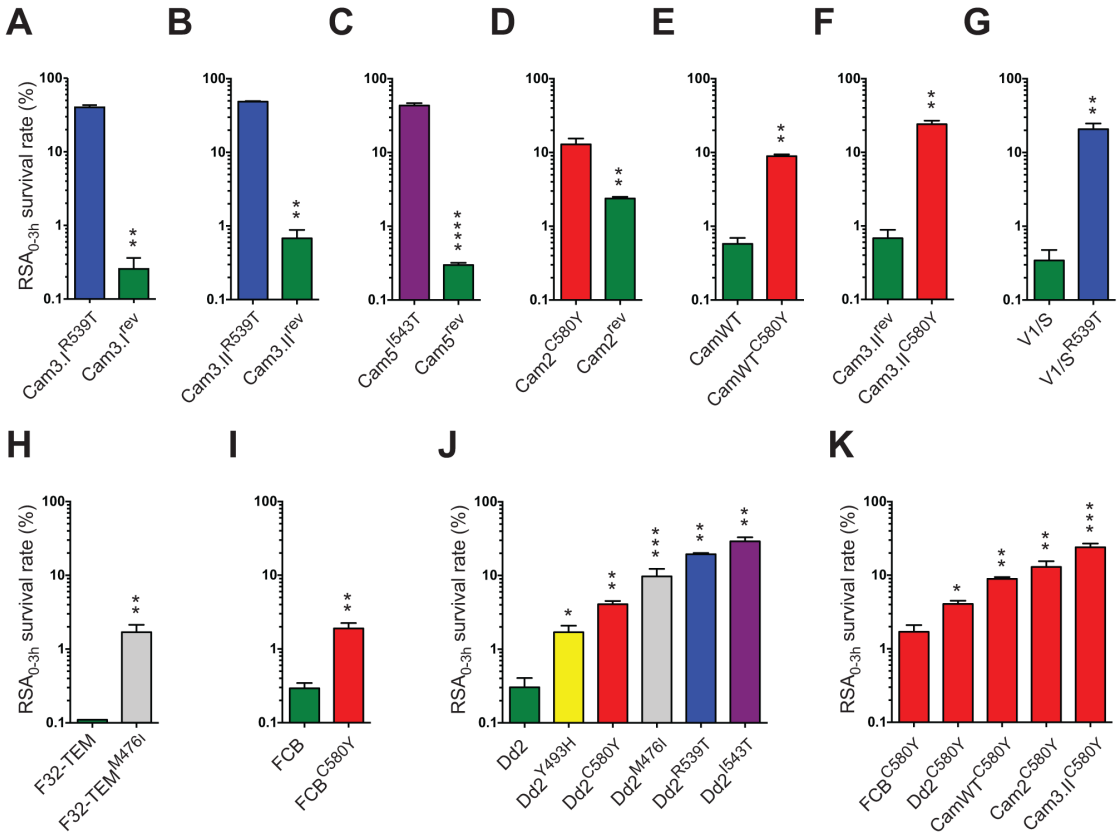

Fig. 2. K13-propeller mutations confer artemisinin resistance in clinical isolates and reference lines in vitro, as defined in the Ring-stage Survival Assay ($RSA_{0\text{-}3h}$). Results show the percentage of early ring-stage parasites (0–3 hours post-invasion of human erythrocytes) that survived a 6-hour pulse of 700 nM DHA (a pharmacologically relevant concentration of the active metabolite of ARTs), as measured by microscopy 66 hours later. Data show mean ± SEM percent survival compared to DMSO-treated parasites processed in parallel. (**A–D**) $RSA_{0\text{-}3h}$ survival for Cambodian isolates harboring native K13 mutations (shown in superscript) and ZFN-edited isogenic clones carrying wild-type *K13* alleles (superscript "rev"). (**E–I**) $RSA_{0\text{-}3h}$ survival for Cambodian isolates and reference lines harboring wild-type *K13* alleles and ZFN-edited isogenic clones carrying individual K13 mutations (shown in superscripts). (**J**) Impact of different K13 mutations on $RSA_{0\text{-}3h}$ survival in the Dd2 reference line, showing that I543T and R539T confer the highest levels of resistance. (**K**) Introduction of C580Y into multiple Cambodian clinical isolates and reference lines, showing that this mutation confers varying degrees of in vitro resistance depending on the parasite genetic background. The geographic origins and known drug-resistance genotypes of these isolates and lines are provided in table S2. Results were obtained from 3–4 independent assays performed in duplicate (values provided in table S4; F32-TEM showed <0.2% $RSA_{0\text{-}3h}$ survival). Two-sample *t* tests with unequal variances (performed using the STATA package) were used to assess for statistically significant differences between *K13*-edited clones and their comparator lines, i.e., the parental isolates listed on the left in panels **A–J** and the FCB^{C580Y} clone in panel **K** (*p<0.05; **p<0.01; ***p<0.001; ****p<0.0001). Statistical outputs (including calculations of the standard error of the difference between the means of samples being compared and the p values) are listed in the Supplementary Materials.

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Supplementary Materials for

K13-propeller mutations confer artemisinin resistance in *Plasmodium falciparum* clinical isolates

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MATERIALS AND METHODS

Zinc-finger nuclease (ZFN) engineering. ZFNs directed against *K13* (PlasmoDB ID PF3D7_1343700; also referred to as Kelch13) were designed and assembled using an archive of validated modules (*33*). These were screened for activity using an episomal reporter system, yielding ZFN pairs 18/19 and 18/20 (**Table S1**).

Plasmid construction. Left and right ZFNs were first linked with the 2A peptide to ensure equal levels of expression driven by a single promoter (*20*). This ZFN L-2A-ZFN R sequence was cloned into the AvrII and XhoI restriction sites in pDC2 (*34*). This placed the fused sequence under the regulatory control of the 5' *calmodulin* (PF3D7_1434200) promoter and the 3' *hsp86* (PF3D7_0708500) terminator. This plasmid also contained the human *dhfr* selectable marker (**Fig. S1**).

We generated six different donor plasmids to introduce either the wild-type *K13* allele or one of five mutant alleles: M476I (allele 1), C580Y (allele 2), R539T (allele 3), Y493H (allele 4) or I543T (allele 5). In a first step we amplified a 1.5-kb donor sequence of the *K13*-propeller domain using the primer pair p1/p2 (**Table S5**) and cloned this into the pGEM®-T vector (Promega). At the zinc-finger binding site we introduced six silent mutations by site-directed mutagenesis using the primer pair p3/p4. Allele-specific mutations were introduced via mutagenesis with specific primer pairs: p5/p6 for M476I, p7/p8 for C580Y, p9/p10 for R539T, p11/p12 for Y493H and p13/p14 for I543T. *K13* donor sequences were excised from the pGEM-T vector with the restriction enzymes SacII and PstI while the ZFN-containing plasmid was digested with SalI and PstI. The two incompatible sites SacII and SalI from the donor fragment and the vector, respectively, were previously filled in using Klenow DNA polymerase. *K13* templates were ligated into the pDC2-based plasmids described above (with primer pairs 18/19 or 18/20, both showing similar efficiency; **Table S1**) using the compatible PstI site and blunt-end cloning, yielding the final pZFN^{K13}-h*dhfr* transfection plasmids.

Parasite cultures, transfections and DNA analysis. Asexual blood-stage parasites were propagated in human erythrocytes in RPMI-1640 medium containing 2 mM L-glutamine, 50 mg/L hypoxanthine, 25 mM HEPES, 0.225% $NaHCO_3$, 10 mg/L gentamycin and 0.5% (w/v) Albumax II (Invitrogen) for the Dd2, FCB and V1/S reference lines. Cambodian isolates and F32 parasites were further supplemented with 5% human AB$^+$ serum (Interstate Blood Bank). Parasites were maintained at 37°C in an atmosphere

of 5% O_2, 5% CO_2 and 90% N_2, and electroporated with purified circular plasmid DNA as described (*35*). One day after electroporation, parasites were exposed to 2.5 nM or 10 nM WR99210 for 6 days (the latter concentration was reserved for quadruple *dhfr* mutant parasites; **Table S3**) to select for transformed parasites (**Fig. S1A**). Parasites generally became microscopically detectable 15–25 days post-electroporation. *K13* editing was assessed by PCR analysis of bulk cultures, and cultures showing the highest level of editing were selected for 96-well cloning by limiting dilution (**Table S2**). Clones were identified after 17–28 days by staining with 2× SYBR Green I and 1.6 μM MitoTracker Deep Red (Invitrogen) and assaying for growth by flow cytometry on an Accuri C6 cytometer (*36*). To test for *K13* editing, *P. falciparum* trophozoite-infected erythrocytes were harvested and saponin-lysed. Parasite genomic DNA was extracted and purified using DNeasy Blood kits (Qiagen). *K13* sequences (**Fig. 1**) were examined by PCR-amplifying the genomic locus with primers p15/p16 flanking the *K13* plasmid donor sequence. These products were amplified from bulk cultures or parasite clones. Sequencing was performed with the internal p17 primer (**Table S5**). For select clones, expression of the mutant or wild-type *K13* alleles was confirmed by RT-PCR and sequencing.

Ring-stage survival assays ($RSA_{0\text{-}3h}$). These assays were carried out as previously described, with minor modifications (*12*). A detailed protocol is available on the WorldWide Antimalarial Resistance Network's website at http://www.wwarn.org/toolkit/procedures/ring-stage-survival-assays-rsa-evaluate-susceptibility-p-falciparum. In summary, 10–15 mL parasite cultures were synchronized 1–2 times using 5% sorbitol (Sigma-Aldrich). Synchronous multinucleated schizonts were incubated in RPMI-1640 containing 15 units/ml sodium heparin for 15 min at 37°C to disrupt agglutinated erythrocytes, concentrated over a gradient of 75% Percoll (Sigma-Aldrich), washed once in RPMI-1640, and incubated for 3 hours with fresh erythrocytes to allow time for merozoite invasion. Cultures were then subjected again to sorbitol treatment to eliminate remaining schizonts. The 0–3 hours post-invasion rings were adjusted to 1% parasitemia and 2% hematocrit in 1 mL volumes (in 48-well plates), and exposed to 700 nM DHA or 0.1% DMSO (solvent control) for 6 hours. Duplicate wells were established for each parasite line ± drug. One-mL cultures were then transferred to 15 mL conical tubes, centrifuged at 800xg for 5 min to pellet the cells, and the supernatants carefully removed. As a washing step to remove drug, 9 mL culture medium were added to each tube and the cells resuspended, centrifuged, and the medium aspirated. Fresh medium lacking drug was then added to cultures, which were returned to standard culture conditions for 66 hours.

Parasite viability was assessed by microscopic examination of Giemsa-stained thin blood smears by counting parasites that developed into second-generation rings or trophozoites with normal morphology. To obtain a homogenous smear for all slides, the cultures were resuspended, transferred into an Eppendorf tube, and briefly centrifuged. 2 μL of the pellet were then used for each smear. Parasitemias were calculated from a total of at least 40,000 erythrocytes per assay. Slides were read from the two duplicate wells per assay by two separate microscopists, each of whom examined at least 10,000 erythrocytes per slide. In instances of >20% discrepancy in parasite counts, slides were further examined by a third microscopist. The mean±SEM numbers of erythrocytes counted per parasite line and per assay were 44,160±970. Our mean±SEM parasitemia for non DHA-treated cultures was 5.1±0.3%. For assays where ≤5 infected erythrocytes were counted from the initial examination of 40,000 erythrocytes (corresponding to a calculated parasitemia of ≤0.0125%), the total number of erythrocytes was expanded to a mean±SEM of 58,360±3,460.

Percent survival was calculated as the parasitemia in the drug-treated sample divided by the parasitemia in the untreated sample ×100. As an example, a 20% $RSA_{0\text{-}3h}$ survival value corresponded to a 1% parasitemia in drug-treated parasites compared to a 5% parasitemia in the untreated control (the mean parasitemia in our untreated controls). Assuming a total cell count of 40,000 erythrocytes, this produced a 95% confidence interval (CI) of 0.9–1.1% parasitemia (corresponding to 360–440 infected erythrocytes for that particular line in one assay). A 2% $RSA_{0\text{-}3h}$ survival value corresponded to a 0.1% parasitemia in the drug-treated sample (95% CI 0.07–0.13%; i.e. 28–52 infected erythrocytes) assuming a count of 40,000 erythrocytes. Given a 0.01% parasitemia (corresponding to a 0.2% $RSA_{0\text{-}3h}$ survival value) and an average count of 58,360 erythrocytes, the 95% CI was 0.002–0.018% (i.e. 1–10 infected erythrocytes). The limit of assay sensitivity was therefore set to 0.2% survival. For each parent and corresponding *K13*-edited clones, individual groups were designated to perform the $RSA_{0\text{-}3h}$ assays, whose results are presented in Fig. 2 and summarized in Table S4.

***In vitro* 50% inhibitory concentration (IC_{50}) assays.** *In vitro* IC_{50} values were determined by incubating parasites for 72 hours across a range of concentrations of DHA or artesunate (0.25–24 nM for both). Proliferation was determined by staining parasites with SYBR Green I and MitoTracker Deep Red, and measuring parasitemia by flow cytometry (see above). *In vitro* IC_{50} values were calculated by nonlinear regression analysis, and Student *t* tests were used for statistical analysis.

Fig. S1

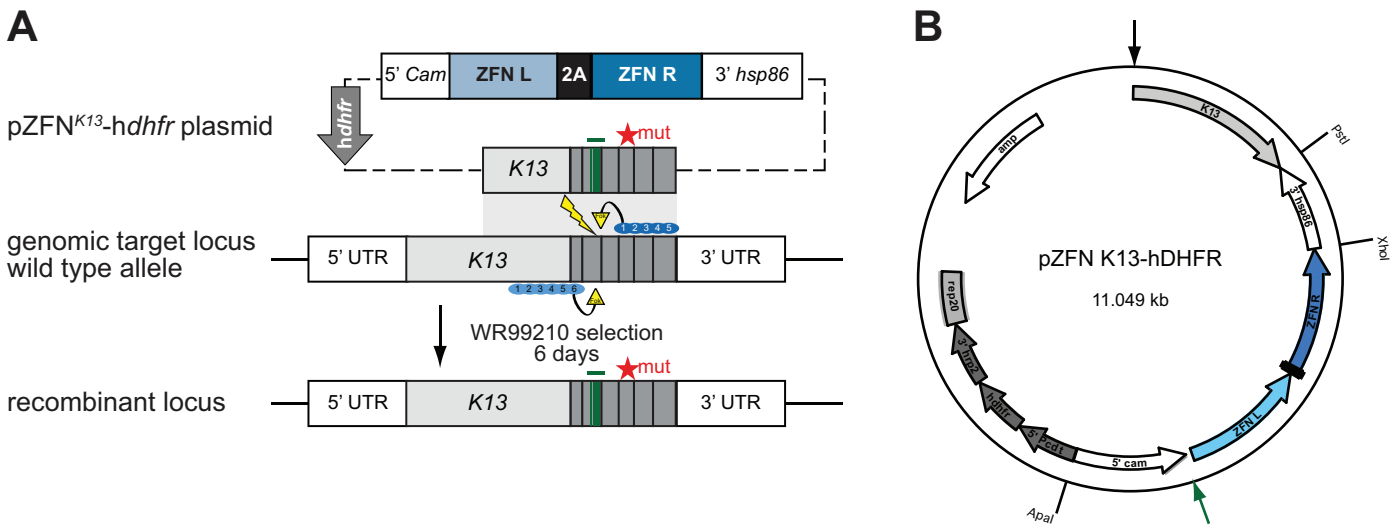

Fig. S1. *K13*-propeller editing strategy and plasmid map. (A) SNPs were inserted into wild-type or removed from mutant *K13*-propeller alleles using zinc-finger nucleases (ZFNs; **Table S1**). In this schematic, ZFNs introduce a double-stranded break (thunderbolt) in the 2nd propeller domain of the wild-type genomic target locus, thus triggering DNA resection. The pZFN^{K13}-h*dhfr* donor plasmid sequence provides a DNA template for homology-directed repair processes. Green bars indicate the presence of silent binding-site mutations that prevent the plasmid and the edited recombinant locus from being cleaved by ZFNs. Red stars indicate the presence of a non-synonymous propeller mutation in the plasmid and edited recombinant locus. **(B)** pZFN^{K13}-h*dhfr* plasmid map. Plasmids contained either the ZFN pairs 18/20 or 19/20 (**Table S1**). Black and green arrows indicate SacII/SalI and AvrII/NheI restriction sites that were ablated during plasmid construction (see supplementary text).

Fig. S2

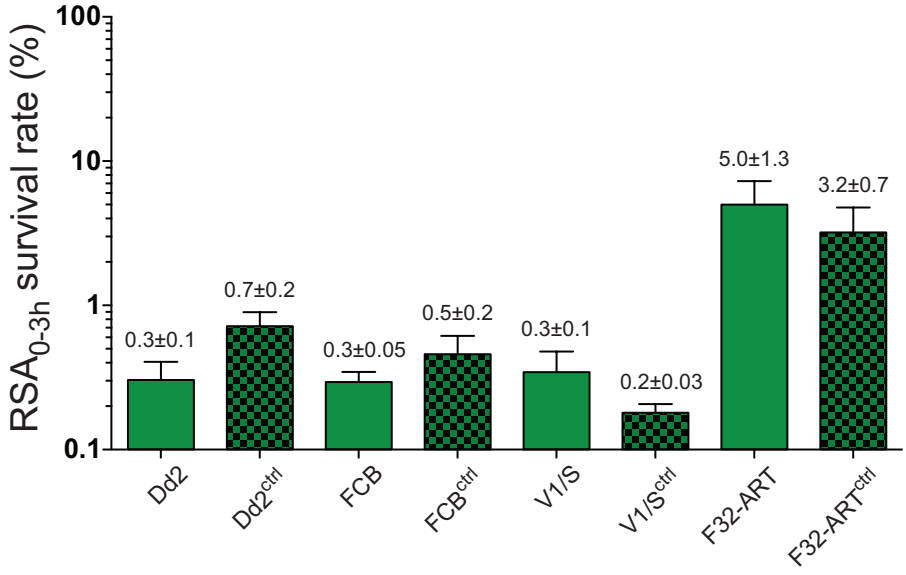

Fig. S2. Synonymous *K13* binding-site mutations do not significantly alter ring-stage susceptibility to artemisinins. Parasite lines were transfected with pZFN^{K13}-h*dhfr* plasmids harboring only silent binding-site mutations in the *K13* donor template (**Fig. 1**), and edited clones were obtained. $RSA_{0\text{-}3h}$ assays were performed on 3–5 separate occasions in duplicate, and percent survival was calculated as described in Methods. We note that Dd2, FCB and V1/S harbor the wild-type *K13* allele, whereas F32-ART harbors the K13-propeller mutation M476I that was acquired during several years of artemisinin pressure *in vitro*. The F32-ART line and the edited F32-ART^{ctrl} line carrying silent binding-site mutations (in addition to M476I), showed survival rates in the $RSA_{0\text{-}3h}$ that were 2–3 fold lower than those originally reported for F32-ART, suggesting that the resistance phenotype had attenuated over time. Mean±SEM values are represented above each column. Student *t* tests revealed no significant differences between the edited clones harboring only the synonymous binding-site mutations (designated with superscript ctrl) and the isogenic parental lines (p>0.05 in all cases).

Fig. S3

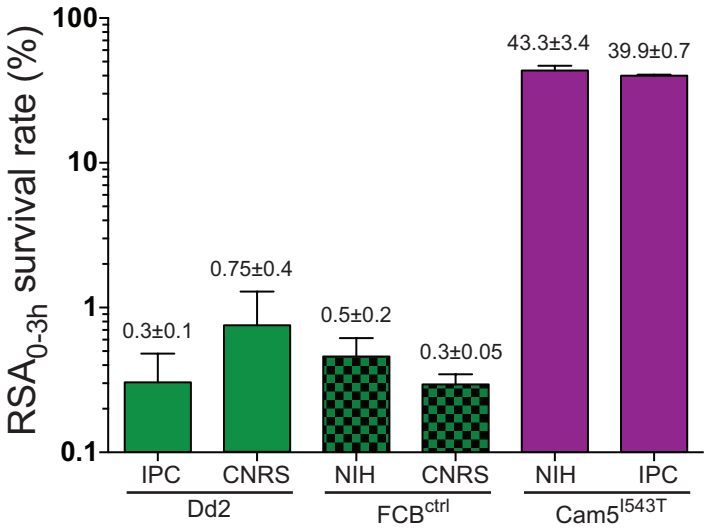

Fig. S3. Demonstration of consistent $RSA_{0\text{-}3h}$ values between test sites. To test for consistency between research groups, several parasite lines were shipped to separate laboratories for independent analysis. Results showed equivalent survival rates among the three sites, namely those led by Dr. Didier Ménard at the Institut Pasteur du Cambodge (IPC), Dr. Françoise Benoit-Vical at the Université de Toulouse, Centre National de Recherche Scientifique UPR8241 (CNRS), and Dr. Rick Fairhurst at the National Institutes of Health (NIH). Assays were performed on 2–3 separate occasions in duplicate, and results are shown as mean±SEM above each column. Dd2 and FCB^{ctrl} parasites harbor the wild-type *K13* allele (the latter parasite line also contains synonymous binding-site mutations), whereas Cam5 parasites harbor the K13 I543T mutation that confers elevated survival rates in the $RSA_{0\text{-}3h}$, indicative of high-level *in vitro* resistance. *t* tests showed no significant difference between $RSA_{0\text{-}3h}$ results of different laboratories when testing the same parasite lines (p>0.05 in all cases).

Fig. S4

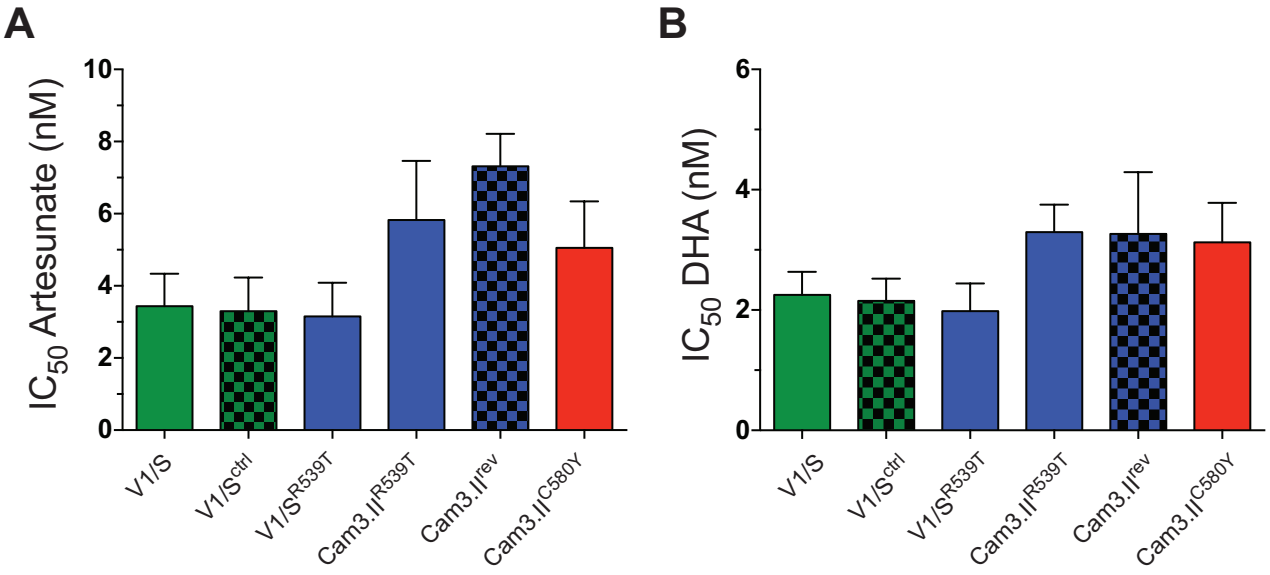

Fig. S4. ***In vitro*** **proliferation assays show no significant differences in IC_{50} values between K13 wild-type and mutant parasites.** IC_{50} values (mean±SEM) were measured in 72-h proliferation assays with final parasitemias determined using flow cytometry with SYBR Green I and MitroTracker Deep Red-stained parasites. Assays were performed on three separate occasions in duplicate. DHA, dihydroartemisinin.

Table S1. Nucleotide sequences of *K13*-specific ZFN binding sites and amino acid sequences of zinc-finger proteins.

K13 ZFN Binding Sequence (underlined)	ZFN	Finger 1	Finger 2	Finger 3	Finger 4	Finger 5	Finger 6
CTTTGACTCCACATACTAGCAAATTCTCTACATACCATACAAAGT	43418-ELD[a]	RSDNLSV	IRSTLRD	RSADLTR	TNQNRIT	TSGNLTR	DSSNLAT
GAAACTGAGGTGTATGATCGTTTAAGAGATGTATGGTATGTTTCA	43419-KKR	TSGSLTR	TSSNRKT	RPTRMQ	QSGSLTR	TSANLSR	-
GAAACTGAGGTGTATGATCGTTTAAGAGATGTATGGTATGTTTCA	43420-KKR	TSGSLSR	TSSNRRH	RSAHLTT	QSGSLTR	TSANLSR	-

Amino acid sequences of ZFNs used in this study (individual fingers are underlined).

43418-ELD (414 amino acids)
MDYKDHDGDYKDHDIDYKDDDDKMAPKKKRKVGIHGVPAAMAERPFQCRICMRNFSRSDNLSVHIRTHTGEKPFACDICGRKFAIRSTLRDHTKIHTGSQKPFQCRICMRNFSRSADL
TRHIRTHTGEKPFACDICGRKFATNQNRITHTKIHTGSQKPFQCRICMRNFSTSGNLTRHIRTHTGEKPFACDICGRKFADSSNLATHTKIHLRGSQLVKSELEEKKSELRHKLKYVP
HEYIELIEIARNSTQDRILEMKVMEFFMKVYGYRGKHLGGSRKPDGAIYTVGSPIDYGVIVDTKAYSGGYNLPIGQADEMERYVEENQTRDKHLNPNEWWKVYPSSVTEFKFLFVSGH
FKGNYKAQLTRLNHITNCNGAVLSVEELLIGGEMIKAGTLTLEEVRRKFNNGEINFRSXX

43419-KKR (382 amino acids)
MDYKDHDGDYKDHDIDYKDDDDKMAPKKKRKVGIHGVPAAMAERPFQCRICMRKFATSGSLTRHTKIHTGEKPFQCRICMRNFSTSSNRKTHIRTHTGEKPFACDICGRKFARPTRMQ
HTKIHTGSQKPFQCRICMRNFSQSGSLTRHIRTHTGEKPFACDICGRKFATSANLSRHTKIHLRGSQLVKSELEEKKSELRHKLKYVPHEYIELIEIARNSTQDRILEMKVMEFFMKV
YGYRGKHLGGSRKPDGAIYTVGSPIDYGVIVDTKAYSGGYNLPIGQADEMQRYVKENQTRNKHINPNEWWKVYPSSVTEFKFLFVSGHFKGNYKAQLTRLNRKTNCNGAVLSVEELLI
GGEMIKAGTLTLEEVRRKFNNGEINFXX

43420-KKR (383 amino acids)
MDYKDHDGDYKDHDIDYKDDDDKMAPKKKRKVGIHGVPAAMAERPFQCRICMRNFSTSGSLSRHIRTHTGEKPFACDICGRKFATSSNRRHHTKIHTGSQKPFQCRICMRKFARSAHL
TTHTKIHTGEKPFQCRICMRNFSQSGSLTRHIRTHTGEKPFACDICGRKFATSANLSRHTKIHLRGSQLVKSELEEKKSELRHKLKYVPHEYIELIEIARNSTQDRILEMKVMEFFMK
VYGYRGKHLGGSRKPDGAIYTVGSPIDYGVIVDTKAYSGGYNLPIGQADEMQRYVKENQTRNKHINPNEWWKVYPSSVTEFKFLFVSGHFKGNYKAQLTRLNRKTNCNGAVLSVEELL

[a] The left ZFN 43418-ELD contains six zinc fingers, whereas the two right ZFNs 43419-KKR and 43420-KKR each contain five. Each finger recognizes a 3-nucleotide *K13* sequence. 43418-ELD binds to the *K13* non-coding (-) strand, whereas 43419-KKR and 43420-KKR bind to the same sequence on the coding (+) strand. 43418-ELD / 43419-KKR, and 43418-ELD / 43420-KKR constitute the ZFN pairs 18/19 and 18/20 respectively (Table S3).

Table S2. Geographic origin, native *K13* allele and drug-resistance genotypes of *Plasmodium falciparum* clinical isolates and reference lines.

Parasite	Original ID	Sanger ID	Provider	Geographic origin (year)	$PCt_{1/2}$ (h)	KH group	K13-propeller	PMID #	*pfcrt*	*pfmdr1*	*pfmdr1* CN	*dhfr*	*dhps*
Cam3.I	IPC 5202	N/A	D. Ménard	Battambang, W. Cambodia (2011)	ND	ND	R539T (allele 3)	24352242	Dd2[a]	184F	4	Triple[b]	436A/540E
Cam3.II	RF 967	PH0306-C	R. Fairhurst	Pursat, W. Cambodia (2010)	6.0	3	R539T (allele 3)	22940027	Dd2	184F	1	Triple	436A/540E
Cam5	IPC 4912	N/A	D. Ménard	Mondulkiri, E. Cambodia (2011)	ND	ND	I543T (allele 5)	24352242	Dd2	WT	1	Quadruple	436F/540E/613S
Cam2	IPC 3445	N/A	D. Ménard	Pailin, W. Cambodia (2010)	ND	ND	C580Y (allele 2)	24352242	Dd2	WT	2	Triple	436A/540E
CamWT	RF 915	PH0164-C	R. Fairhurst	Pursat, W. Cambodia (2010)	3.7	1	Wild-type	22940027	Dd2	WT	1	Triple	436A/540E
F32-TEM	F32	N/A	F. Benoit-Vical	Tanzania (1982)	N/A	N/A	Wild-type	20160056	WT	WT	1	WT	436A
V1/S	V1/S	N/A	MR4	Vietnam (1976)	N/A	N/A	Wild-type	12124623	Dd2	86Y	1	Quadruple	613T
FCB	FCB	N/A	MR4	Asia (1980)	N/A	N/A	Wild-type	12124623	FCB	86Y	2	16V/108T	437A
Dd2	Dd2	N/A	MR4	Indochina (1980)	N/A	N/A	Wild-type	12124623	Dd2	86Y	3	Triple	436F/613S

[a] Dd2: 74I/75D/76T/220S/271E/326S/356T/371I; FCB: 74I/75E/76T/220S/271E/326S/371I.

[b] Triple, 51I/59R/108N; Quadruple, 51I/59R/108N/164L.

$PCt_{1/2}$, parasite clearance half-life; PMID #, PubMed identification number; *pfmdr1* CN, *pfmdr1* copy number; MR4, Malaria Research and Reference Reagent Resource, Manassas, VA; N/A, not available; ND, not determined.

Table S3. *K13* transfection outcomes with *Plasmodium falciparum* clinical isolates and reference lines.

		Binding site mutations only		M476I (allele 1)		C580Y (allele 2)		R539T (allele 3)		Y493H (allele 4)		I543T (allele 5)	
Parasite	**ZFN pair**	Edited cultures (%)	Edited clones (%)	Edited cultures (%)	Edited clones (%)	Edited cultures (%)	Edited clones (%)	Edited cultures (%)	Edited clones (%)	Edited cultures (%)	Edited clones (%)	Edited cultures (%)	Edited clones (%)
Cam3.I	18/19	1 of 1 (100%)	ND	ND	N/A	ND	N/A	ND	N/A	ND	N/A	ND	N/A
Cam3.I	18/20	1 of 1 (100%)	2 of 7 (29%) a	ND	N/A	ND	N/A	ND	N/A	ND	N/A	ND	N/A
Cam3.II	18/19	1 of 2 (50%)	4 of 16 (25%)	ND	N/A	0 of 1 (0%)	ND	ND	N/A	ND	N/A	ND	N/A
Cam3.II	18/20	1 of 2 (50%)	ND	ND	N/A	1 of 1 (100%)	1 of 4 (25%)	ND	N/A	ND	N/A	ND	N/A
Cam5	18/19	1 of 4 (25%)	ND	ND	N/A	ND	N/A	ND	N/A	ND	N/A	ND	N/A
Cam5	18/20	1 of 4 (25%)	6 of 11 (55%)	ND	N/A	ND	N/A	ND	N/A	ND	N/A	ND	N/A
Cam2	18/19	1 of 2 (50%)	1 of 6 (17%)	ND	N/A	ND	N/A	ND	N/A	ND	N/A	ND	N/A
Cam2	18/20	1 of 2 (50%)	ND	ND	N/A	ND	N/A	ND	N/A	ND	N/A	ND	N/A
CamWT	18/19	0 of 1 (0%)	ND	ND	N/A	1 of 2 (50%)	1 of 2 (50%)	0 of 1 (0%)	ND	0 of 2 (0%)	ND	1 of 2 (50%)	0 of 3 (0%)
CamWT	18/20	ND	N/A	ND	N/A	0 of 2 (0%)	ND	0 of 1 (0%)	ND	1 of 2 (50%)	ND	ND	N/A
F32-TEM	18/19	ND	N/A	1 of 2 (50%)	ND	ND	N/A	ND	N/A	ND	ND	ND	N/A
F32-TEM	18/20	ND	N/A	1 of 2 (50%)	2 of 19 (11%)	ND	N/A	ND	N/A	ND	N/A	ND	N/A
F32-ART	18/19	1 of 2 (50%)	N/A	ND	N/A	ND	N/A	ND	N/A	ND	N/A	ND	N/A
F32-ART	18/20	1 of 2 (50%)	0 of 3 (0%) b	ND	N/A	ND	N/A	ND	N/A	ND	N/A	ND	N/A
V1/S	18/19	0 of 1 (0%)	N/A	0 of 2 (0%)	ND	1 of 1 (100%)	0 of 18 (0%)	0 of 1 (0%)	ND	1 of 2 (50%)	ND	0 of 1 (0%)	ND
V1/S	18/20	1 of 1 (100%)	ND c	0 of 2 (0%)	ND	0 of 1 (0%)	ND	1 of 1 (100%)	1 of 8 (13%)	0 of 1 (0%)	ND	0 of 1 (0%)	ND
FCB	18/19	0 of 1 (0%)	N/A	0 of 1 (0%)	ND	1 of 2 (50%)	2 of 25 (8%)	0 of 3 (0%)	ND	0 of 2 (0%)	ND	0 of 2 (0%)	ND
FCB	18/20	0 of 1 (0%)	N/A	0 of 1 (0%)	ND	0 of 2 (0%)	ND	0 of 3 (0%)	ND	0 of 3 (0%)	ND	0 of 2 (0%)	ND
Dd2	18/19	0 of 1 (0%)	N/A	0 of 3 (0%)	ND	1 of 1 (100%)	1 of 10 (10%)	0 of 6 (0%)	ND	1 of 1 (100%)	1 of 17 (6%)	0 of 3 (0%)	ND
Dd2	18/20	1 of 3 (33%)	ND	1 of 3 (33%)	1 of 8 (13%)	0 of 1 (0%)	ND	1 of 6 (0%)	1 of 8 (13%)	0 of 1 (0%)	ND	1 of 3 (33%)	3 of 21 (14%)
Positive events		12 of 30 (40%) d	13 of 43 (30%)	3 of 16 (19%)	3 or 27 (11%)	5 of 14 (36%) e	5 of 59 (9%)	2 of 22 (9%)	2 of 16 (13%)	3 of 14 (21%)	1 of 17 (6%)	2 of 14 (14%)	3 of 24 (13%)

[a] Clones were generated from individual bulk cultures with the highest percentage of editing events for a given mutation and parasite.The percentages of edited clones are listed only for those that captured the desired nucleotide changes (either insertion or removal of a point mutation of interest). Clones that captured binding site mutations but not *K13* mutations were not included in this analysis; therefore, the percentages listed underestimate the percentage of editing events.

[b] One clone (F32-ART^{ctrl} in Fig. S3) captured the binding site mutations, but did not revert M476I to wild-type. This clone was therefore not listed as being edited at the mutation of interest (see definition in footnote a).

[c] This culture was not cloned because the desired binding site control line (V1/S^{ctrl}) was obtained from the transfection with the R539T plasmid, resulting in incorporation of the binding site mutations but not R539T. This was one of several instances where a binding site mutant control was obtained from transfections with mutant donor sequences that captured only the binding site mutations, but not the *K13* mutations more distant from the ZFN cleavage site. Binding site control clones are shown in Fig. S3.

[d] These data suggest that removing K13 mutations (achieved using the plasmid containing the binding site mutations only) was generally more efficient than introducing them, possibly due to a fitness cost associated with their presence.

[e] These data suggest that C580Y editing into K13 wild-type parasites was more efficient than editing of other mutations, possibly because the C580Y mutation might have a lesser fitness cost. These results are consistent with C580Y being highly prevalent in western Cambodia, despite our evidence that C580Y mediates a lower level of *in vitro* resistance than R539T or I543T (see Fig. 2).

N/A, not applicable; ND, not done.

Table S4. Data from ring-stage survival assays (RSAs).

Parasite	Original isolate/line	K13 sequence	Binding-site mutations	RSA values mean ± SEM	Assays	Primary site of RSA data
Cam3.I^{R539T}	IPC 5202	R539T (parent allele 3)	No	40.2 ± 3.0[a,b]	3[c]	IPC, Cambodia[d]
Cam3.I^{rev}	IPC 5202	wild-type (revertant)	Yes	0.3 ± 0.1	3	IPC, Cambodia
Cam3.II^{R539T}	RF 967	R539T (parent allele 3)	No	48.9 ± 0.8	3	NIH, USA
Cam3.II^{rev}	RF 967	wild-type (revertant)	Yes	0.7 ± 0.2	3	NIH, USA
Cam3.II^{C580Y}	RF 967	C580Y (allele 2)	Yes	24.1 ± 2.9	3	NIH, USA
Cam5^{I543T}	IPC 4912	I543T (parent allele 5)	No	43.4 ± 3.4	3	NIH, USA
Cam5^{rev}	IPC 4912	wild-type (revertant)	Yes	0.3 ± 0.02	3	NIH, USA
Cam2^{C580Y}	IPC 3445	C580Y (parent allele 2)	No	13.0 ± 2.6	3	IPC, Cambodia
Cam2^{rev}	IPC 3445	wild-type (revertant)	Yes	2.4 ± 0.1	3	IPC, Cambodia
CamWT	RF 915	wild-type (parent)	No	0.6 ± 0.1	3	NIH, USA
CamWT^{C580Y}	RF 915	C580Y (allele 2)	Yes	8.9 ± 0.5	3	NIH, USA
F32-TEM	F32-TEM	wild-type (parent)	No	<0.2[e]	4	CNRS, France
F32-TEM^{M476I}	F32-TEM	M476I (allele 1)	Yes	1.7 ± 0.4	4	CNRS, France
V1/S	V1/S	wild-type (parent)	No	0.3 ± 0.1	3	NIH, USA
V1/S^{R539T}	V1/S	R539T (allele 3)	Yes	20.7 ± 4.0	4	NIH, USA
FCB	FCB	wild-type (parent)	No	0.3 ± 0.1	3	NIH, USA
FCB^{C580Y}	FCB	C580Y (allele 2)	Yes	1.9 ± 0.3	4	NIH, USA
Dd2	Dd2	wild-type (parent)	No	0.3 ± 0.1	3	IPC, Cambodia
Dd2^{Y493H}	Dd2	Y493H (allele 4)	Yes	1.7 ± 0.4	3	IPC, Cambodia
Dd2^{C580Y}	Dd2	C580Y (allele 2)	Yes	4.1 ± 0.4	3	IPC, Cambodia
Dd2^{M476I}	Dd2	M476I (allele 1)	Yes	9.8 ± 2.6	4	CNRS, France
Dd2^{R539T}	Dd2	R539T (allele 3)	Yes	19.4 ± 0.8	3	NIH, USA
Dd2^{I543T}	Dd2	I543T (allele 5)	Yes	29.1 ± 3.9	3	IPC, Cambodia

[a] RSA values are the percentage of parasites that survived a 6-h pulse of 700 nM dihydroartemisinin, compared to DMSO-treated parasites. Parasitemias were determined 66 h after ending the drug pulse.

[b] For each RSA value, the relative standard error (RSE) was calculated as the SEM/mean x100. The mean RSE for all assays was 17.0%, indicating an average SEM of 17.0% of the mean.

[c] Assays were performed on separate occasions as enumerated, with each assay performed in duplicate.

[d] IPC, Institut Pasteur du Cambodge (Ménard lab); CNRS, Centre National de Recherche Scientifique UPR8241 (Benoit-Vical lab); NIH, National Institutes of Health (Fairhurst lab).

[e] RSA values for F32-TEM were calculated to be 0.02±0.02. Based on our limit of sensitivity (as discussed in Supplementary Materials) we have indicated this survival value to be <0.2%.

Table S5. Oligonucleotides used in this study.

Name	Nucleotide sequence (5'-3')[a]	Description	Lab name
p1	GTGACGTCGATTGATATTAATGTTGGTGGAGC	*K13* donor fwd	p3984
p2	CCGCATATGGTGCAAACGGAGTGACCAAATCTGGG	*K13* donor rev	p3986
p3	GGTGTATGATaGaTTAAGAGAcGTcTGGTATGTaTCAAGTAATTTAAATATACC	SDM ZFN binding site mutation fwd	p4169
p4	CCAgACgTCTCTTAAtCtATCATACACCTCtGTTTCAAATAAAGCC	SDM ZFN binding site mutation rev	p4170
p5	GCTGGCGTATGTGTACACCTATaTCTACCAAAAAAGCTTATTTTGGAAGTGC	SDM allele 1 M476I fwd	p4173
p6	GGTAGAtATAGGTGTACACATACGCCAGCATTGTTGACTAATATCTAATAATTCC	SDM allele 1 M476I rev	p4174
p7	CATCAGCTATGTaTGTTGCTTTTGATAATAAAATTTATGTCATTGG	SDM allele 2 C580Y fwd	p4003
p8	ATTATCAAAAGCAACAtACATAGCTGATGATCTAGGGGTATTCAAAGG	SDM allele 2 C580Y rev	p4004
p9	CGTCAAATGGTAcAATTTATTGTATTGGGGGATATGATGGCTCTTC	SDM allele 3 R539T fwd	p4005
p10	CCAATACAATAAATTgTACCATTTGACGTAACACCACAATTATTTC	SDM allele 3 R539T rev	p4006
p11	GAATAATTTCTTAcACGTTTTTGGTGGTAATAACTATGATTATAAGGC	SDM allele 4 Y493H fwd	p4188
p12	CCAAAAACGTgTAAGAAATTATTCAATACAGCACTTCCAAAATAAGC	SDM allele 4 Y493H rev	p4189
p13	GGTAGAATTTATTGTAcTGGGGGATATGATGGCTCTTCTATTATACC	SDM allele 5 I543T fwd	p4007
p14	CATATCCCCCAgTACAATAAATTCTACCATTTGACGTAACACCAC	SDM allele 5 I543T rev	p4008
p15	GCAAATCTTATAAATGATGATTCTGG	*K13* 5' for screening fwd	p4433
p16	GCTAATAAGTAATATCAATATAAGGG	*K13* 3' for screening rev	p4434
p17	GGTATTAAATTTTTACCATTCCCATTAGTATTTTGTATAGG	Sequencing primer	p4186

[a] Lower-case nucleotides show the position that was subjected to mutagenesis.

fwd, forward; rev, reverse; SDM, site directed mutagenesis.

Statistical data: Results of two-sample t-tests with unequal variances

Cam3.I^{R539T} vs. Cam3.I^{rev} (groups 1 vs. 2 respectively; p<0.01; Fig. 2A)

```
. ttest logCam3I, by(group) unequal
```

Two-sample t test with unequal variances

Group	Obs	Mean	Std. Err.	Std. Dev.	[95% Conf.	Interval]
1	3	3.688271	.0768044	.133029	3.357809	4.018734
2	3	-1.546587	.4405906	.7631253	-3.442296	.3491212
combined	6	1.070842	1.187515	2.908805	-1.981761	4.123445
diff		5.234858	.4472348		3.412362	7.057355

```
    diff = mean(1) - mean(2)                                      t =  11.7049
Ho: diff = 0                     Satterthwaite's degrees of freedom =  2.12144

    Ha: diff < 0                 Ha: diff != 0                 Ha: diff > 0
 Pr(T < t) = 0.9971         Pr(|T| > |t|) = 0.0058          Pr(T > t) = 0.0029
```

Cam3.II^{R539T} vs. Cam3.II^{rev} (groups 1 vs. 2 respectively; p<0.01; Fig. 2B)

```
. ttest logCam3II, by(group) unequal
```

Two-sample t test with unequal variances

Group	Obs	Mean	Std. Err.	Std. Dev.	[95% Conf.	Interval]
1	3	3.89019	.016374	.0283606	3.819738	3.960642
2	3	-.5074539	.3730999	.646228	-2.112773	1.097866
combined	6	1.691368	.9974256	2.443184	-.8725962	4.255332
diff		4.397644	.3734591		2.796674	5.998614

```
    diff = mean(1) - mean(2)                                      t =  11.7754
Ho: diff = 0                     Satterthwaite's degrees of freedom =   2.0077

    Ha: diff < 0                 Ha: diff != 0                 Ha: diff > 0
 Pr(T < t) = 0.9965         Pr(|T| > |t|) = 0.0070          Pr(T > t) = 0.0035
```

$Cam5^{I543T}$ vs. $Cam5^{rev}$ (groups 1 vs. 2 respectively; p=<0.0001; Fig. 2C)

```
. ttest logCam5, by(group) unequal
```

Two-sample t test with unequal variances

Group	Obs	Mean	Std. Err.	Std. Dev.	[95% Conf.	Interval]
1	3	3.763479	.0753753	.1305539	3.439165	4.087793
2	3	-1.215887	.0725464	.125654	-1.528029	-.9037451
combined	6	1.273796	1.114403	2.729718	-1.590867	4.138459
diff		4.979366	.1046155		4.688739	5.269993

```
    diff = mean(1) - mean(2)                                      t =  47.5968
Ho: diff = 0                     Satterthwaite's degrees of freedom =  3.99416

    Ha: diff < 0                 Ha: diff != 0                 Ha: diff > 0
 Pr(T < t) = 1.0000         Pr(|T| > |t|) = 0.0000          Pr(T > t) = 0.0000
```

$Cam2^{C580Y}$ vs. $Cam2^{rev}$ (groups 1 vs. 2 respectively; p<0.01; Fig. 2D)

```
. ttest logCam2, by(group) unequal
```

Two-sample t test with unequal variances

Group	Obs	Mean	Std. Err.	Std. Dev.	[95% Conf.	Interval]
1	3	2.525393	.1843995	.3193893	1.731986	3.3188
2	3	.867163	.0518709	.0898431	.6439804	1.090346
combined	6	1.696278	.3805589	.9321752	.7180201	2.674536
diff		1.65823	.1915562		.9325863	2.383874

```
    diff = mean(1) - mean(2)                                      t =   8.6566
Ho: diff = 0                     Satterthwaite's degrees of freedom =  2.31454

    Ha: diff < 0                 Ha: diff != 0                 Ha: diff > 0
 Pr(T < t) = 0.9959         Pr(|T| > |t|) = 0.0082          Pr(T > t) = 0.0041
```

CamWT vs. CamWT^{C580Y} (groups 1 vs. 2 respectively; p<0.01; Fig. 2E)

```
. ttest logCamWT, by(group) unequal
```

Two-sample t test with unequal variances

Group	Obs	Mean	Std. Err.	Std. Dev.	[95% Conf.	Interval]
1	3	-.5885569	.1905804	.330095	-1.408558	.2314445
2	3	2.184728	.0602016	.1042722	1.925701	2.443754
combined	6	.7980854	.6265337	1.534688	-.8124706	2.408642
diff		-2.773285	.1998628		-3.510595	-2.035975

```
    diff = mean(1) - mean(2)                                      t = -13.8759
Ho: diff = 0                     Satterthwaite's degrees of freedom =   2.3952

    Ha: diff < 0                 Ha: diff != 0                 Ha: diff > 0
 Pr(T < t) = 0.0012         Pr(|T| > |t|) = 0.0024          Pr(T > t) = 0.9988
```

Cam3.II^{rev} vs. Cam3.II^{C580Y} (groups 1 vs. 2 respectively; p<0.01; Fig. 2F)

```
. ttest logCam3IIC580Y, by(group) unequal
```

Two-sample t test with unequal variances

Group	Obs	Mean	Std. Err.	Std. Dev.	[95% Conf.	Interval]
1	3	-.5074539	.3730999	.646228	-2.112773	1.097866
2	3	3.167337	.1149281	.1990612	2.672841	3.661832
combined	6	1.329941	.8400516	2.057698	-.8294799	3.489363
diff		-3.67479	.3903998		-5.123775	-2.225806

```
    diff = mean(1) - mean(2)                                      t =  -9.4129
Ho: diff = 0                     Satterthwaite's degrees of freedom =  2.37616

    Ha: diff < 0                 Ha: diff != 0                 Ha: diff > 0
 Pr(T < t) = 0.0031         Pr(|T| > |t|) = 0.0062          Pr(T > t) = 0.9969
```

V1/S vs. V1/S^{R539T} (groups 1 vs. 2 respectively; p<0.01; Fig. 2G)

```
. ttest logV1S, by(group) unequal
```

Two-sample t test with unequal variances

Group	Obs	Mean	Std. Err.	Std. Dev.	[95% Conf.	Interval]
1	3	-1.208234	.3612046	.6256247	-2.762372	.3459035
2	4	2.972716	.1999613	.3999226	2.33635	3.609082
combined	7	1.18088	.8622912	2.281408	-.9290704	3.290831
diff		-4.18095	.4128599		-5.447017	-2.914884

```
    diff = mean(1) - mean(2)                                      t = -10.1268
Ho: diff = 0                     Satterthwaite's degrees of freedom =  3.21256

    Ha: diff < 0                 Ha: diff != 0                 Ha: diff > 0
 Pr(T < t) = 0.0008         Pr(|T| > |t|) = 0.0015          Pr(T > t) = 0.9992
```

F32-TEM vs. F32-TEM^{M476I} (groups 1 vs. 2 respectively; p<0.01; Fig. 2H)

```
. ttest logF32TEM, by(group) unequal
```

Two-sample t test with unequal variances

Group	Obs	Mean	Std. Err.	Std. Dev.	[95% Conf.	Interval]
1	3	-1.609438	0	0	-1.609438	-1.609438
2	4	.3982619	.3075268	.6150537	-.5804257	1.37695
combined	7	-.4621809	.4376593	1.157938	-1.533095	.6087328
diff		-2.0077	.3075268		-2.986388	-1.029012

```
    diff = mean(1) - mean(2)                                      t =  -6.5285
Ho: diff = 0                     Satterthwaite's degrees of freedom =       3

    Ha: diff < 0                 Ha: diff != 0                 Ha: diff > 0
 Pr(T < t) = 0.0037         Pr(|T| > |t|) = 0.0073          Pr(T > t) = 0.9963
```

FCB vs. FCB^{C580Y} (groups 1 vs. 2 respectively; p<0.01; Fig. 2I)

```
. ttest logFCB, by(group) unequal
```

Two-sample t test with unequal variances

Group	Obs	Mean	Std. Err.	Std. Dev.	[95% Conf.	Interval]
1	3	-1.260332	.1874178	.3246172	-2.066726	-.4539377
2	4	.5756699	.2217322	.4434645	-.129981	1.281321
combined	7	-.2111879	.3957941	1.047173	-1.179661	.7572853
diff		-1.836002	.2903285		-2.582576	-1.089427

```
    diff = mean(1) - mean(2)                                      t =  -6.3239
Ho: diff = 0                     Satterthwaite's degrees of freedom =  4.99418

    Ha: diff < 0                 Ha: diff != 0                 Ha: diff > 0
 Pr(T < t) = 0.0007         Pr(|T| > |t|) = 0.0015          Pr(T > t) = 0.9993
```

Dd2 vs. Dd2^{Y493H} (groups 1 vs. 2 respectively; p<0.05; Fig. 2J)

```
. ttest logDd2Y493H, by(group) unequal
```

Two-sample t test with unequal variances

Group	Obs	Mean	Std. Err.	Std. Dev.	[95% Conf.	Interval]
1	3	-1.304008	.3314896	.5741568	-2.730292	.1222767
2	3	.4804795	.2148663	.3721593	-.4440154	1.404975
combined	6	-.4117641	.4363832	1.068916	-1.533523	.7099947
diff		-1.784487	.3950352		-2.957299	-.6116756

```
    diff = mean(1) - mean(2)                                      t =  -4.5173
Ho: diff = 0                     Satterthwaite's degrees of freedom =  3.42843

    Ha: diff < 0                 Ha: diff != 0                 Ha: diff > 0
 Pr(T < t) = 0.0076         Pr(|T| > |t|) = 0.0152          Pr(T > t) = 0.9924
```

Dd2 vs. Dd2^{C580Y} (groups 1 vs. 2 respectively; p<0.01; Fig. 2J)

```
. ttest logDd2C580Y, by(group) unequal

Two-sample t test with unequal variances
```

Group	Obs	Mean	Std. Err.	Std. Dev.	[95% Conf.	Interval]
1	3	-1.304008	.3314896	.5741568	-2.730292	.1222767
2	3	1.39194	.1087222	.1883124	.9241457	1.859734
combined	6	.043966	.622694	1.525282	-1.55672	1.644652
diff		-2.695947	.3488637		-3.970905	-1.42099

```
    diff = mean(1) - mean(2)                                      t =  -7.7278
Ho: diff = 0                     Satterthwaite's degrees of freedom =  2.42536

    Ha: diff < 0                 Ha: diff != 0                 Ha: diff > 0
 Pr(T < t) = 0.0046         Pr(|T| > |t|) = 0.0092          Pr(T > t) = 0.9954
```

Dd2 vs. Dd2^{M476I} (groups 1 vs. 2 respectively; p<0.001; Fig. 2J)

```
. ttest logDd2M476I, by(group) unequal

Two-sample t test with unequal variances
```

Group	Obs	Mean	Std. Err.	Std. Dev.	[95% Conf.	Interval]
1	3	-1.304008	.3314896	.5741568	-2.730292	.1222767
2	4	2.162859	.2838199	.5676399	1.259617	3.0661
combined	7	.6770588	.7275241	1.924848	-1.103129	2.457246
diff		-3.466866	.4363933		-4.634165	-2.299568

```
    diff = mean(1) - mean(2)                                      t =  -7.9444
Ho: diff = 0                     Satterthwaite's degrees of freedom =  4.42262

    Ha: diff < 0                 Ha: diff != 0                 Ha: diff > 0
 Pr(T < t) = 0.0004         Pr(|T| > |t|) = 0.0009          Pr(T > t) = 0.9996
```

Dd2 vs. Dd2^{R539T} (groups 1 vs. 2 respectively; p<0.01; Fig. 2J)

```
. ttest logDd2R539T, by(group) unequal
```

Two-sample t test with unequal variances

Group	Obs	Mean	Std. Err.	Std. Dev.	[95% Conf.	Interval]
1	3	-1.304008	.3314896	.5741568	-2.730292	.1222767
2	3	2.96476	.0417134	.0722497	2.785281	3.144238
combined	6	.830376	.966149	2.366572	-1.653189	3.313941
diff		-4.268767	.3341038		-5.664839	-2.872696

```
    diff = mean(1) - mean(2)                                      t = -12.7768
Ho: diff = 0                     Satterthwaite's degrees of freedom =  2.06332

    Ha: diff < 0                 Ha: diff != 0                 Ha: diff > 0
 Pr(T < t) = 0.0027         Pr(|T| > |t|) = 0.0054          Pr(T > t) = 0.9973
```

Dd2 vs. Dd2^{I543T} (groups 1 vs. 2 respectively; p<0.01; Fig. 2J)

```
. ttest logDd2I543T, by(group) unequal
```

Two-sample t test with unequal variances

Group	Obs	Mean	Std. Err.	Std. Dev.	[95% Conf.	Interval]
1	3	-1.304008	.3314896	.5741568	-2.730292	.1222767
2	3	3.353108	.1395851	.2417684	2.752522	3.953694
combined	6	1.02455	1.053713	2.581058	-1.684104	3.733204
diff		-4.657116	.3596795		-5.880846	-3.433385

```
    diff = mean(1) - mean(2)                                      t = -12.9480
Ho: diff = 0                     Satterthwaite's degrees of freedom =  2.68763

    Ha: diff < 0                 Ha: diff != 0                 Ha: diff > 0
 Pr(T < t) = 0.0008         Pr(|T| > |t|) = 0.0017          Pr(T > t) = 0.9992
```

FCB^{C580Y} vs. Dd2^{C580Y} (groups 1 vs. 2 respectively; p<0.05; Fig. 2K)

```
. ttest logDd2C580Y, by(group) unequal
```

Two-sample t test with unequal variances

Group	Obs	Mean	Std. Err.	Std. Dev.	[95% Conf.	Interval]
1	4	.5756699	.2217322	.4434645	-.129981	1.281321
2	3	1.39194	.1087222	.1883124	.9241457	1.859734
combined	7	.9254998	.2071994	.5481982	.418501	1.432499
diff		-.8162698	.2469528		-1.486439	-.1461009

```
    diff = mean(1) - mean(2)                                      t =  -3.3054
Ho: diff = 0                     Satterthwaite's degrees of freedom =  4.24766

    Ha: diff < 0                 Ha: diff != 0                 Ha: diff > 0
 Pr(T < t) = 0.0136         Pr(|T| > |t|) = 0.0272          Pr(T > t) = 0.9864
```

FCB^{C580Y} vs. CamWT^{C580Y} (groups 1 vs. 2 respectively; p<0.01; Fig. 2K)

```
. ttest logCamWTC580Y, by(group) unequal
```

Two-sample t test with unequal variances

Group	Obs	Mean	Std. Err.	Std. Dev.	[95% Conf.	Interval]
1	4	.5756699	.2217322	.4434645	-.129981	1.281321
2	3	2.184728	.0602016	.1042722	1.925701	2.443754
combined	7	1.265266	.3467581	.9174356	.4167797	2.113753
diff		-1.609058	.2297595		-2.290976	-.9271395

```
    diff = mean(1) - mean(2)                                      t =  -7.0032
Ho: diff = 0                     Satterthwaite's degrees of freedom =  3.43063

    Ha: diff < 0                 Ha: diff != 0                 Ha: diff > 0
 Pr(T < t) = 0.0019         Pr(|T| > |t|) = 0.0038          Pr(T > t) = 0.9981
```

FCB^{C580Y} vs. Cam2^{C580Y} (groups 1 vs. 2 respectively; p<0.01; Fig. 2K)

```
. ttest logCam2C580Y, by(group) unequal
```

Two-sample t test with unequal variances

Group	Obs	Mean	Std. Err.	Std. Dev.	[95% Conf.	Interval]
1	4	.5756699	.2217322	.4434645	-.129981	1.281321
2	3	2.525393	.1843995	.3193893	1.731986	3.3188
combined	7	1.411266	.4172107	1.103836	.3903876	2.432143
diff		-1.949723	.2883892		-2.691124	-1.208322

```
    diff = mean(1) - mean(2)                                      t =  -6.7607
Ho: diff = 0                     Satterthwaite's degrees of freedom =  4.99837

    Ha: diff < 0                 Ha: diff != 0                 Ha: diff > 0
 Pr(T < t) = 0.0005         Pr(|T| > |t|) = 0.0011          Pr(T > t) = 0.9995
```

FCB^{C580Y} vs. Cam3.II^{C580Y} (groups 1 vs. 2 respectively; p<0.001; Fig. 2K)

```
. ttest logCam3IIC580Y, by(group) unequal
```

Two-sample t test with unequal variances

Group	Obs	Mean	Std. Err.	Std. Dev.	[95% Conf.	Interval]
1	4	.5756699	.2217322	.4434645	-.129981	1.281321
2	3	3.167337	.1149281	.1990612	2.672841	3.661832
combined	7	1.686384	.5385969	1.424993	.3684851	3.004283
diff		-2.591667	.2497471		-3.263246	-1.920088

```
    diff = mean(1) - mean(2)                                      t = -10.3772
Ho: diff = 0                     Satterthwaite's degrees of freedom =  4.35678

    Ha: diff < 0                 Ha: diff != 0                 Ha: diff > 0
 Pr(T < t) = 0.0002         Pr(|T| > |t|) = 0.0003          Pr(T > t) = 0.9998
```

Dd2 vs. Dd2^{ctrl} (groups 1 vs. 2 respectively; p = not significant; Fig. S2)

```
. ttest logDd2, by(group) unequal
```

Two-sample t test with unequal variances

Group	Obs	Mean	Std. Err.	Std. Dev.	[95% Conf.	Interval]
1	3	-1.304008	.3314896	.5741568	-2.730292	.1222767
2	3	-.3955087	.2486298	.4306395	-1.465276	.6742591
combined	6	-.8497582	.2749708	.6735382	-1.556593	-.1429232
diff		-.908499	.4143695		-2.095421	.2784229

```
    diff = mean(1) - mean(2)                                      t =  -2.1925
Ho: diff = 0                     Satterthwaite's degrees of freedom =  3.70929

    Ha: diff < 0                 Ha: diff != 0                 Ha: diff > 0
 Pr(T < t) = 0.0494         Pr(|T| > |t|) = 0.0988          Pr(T > t) = 0.9506
```

FCB vs. FCB^{ctrl} (groups 1 vs. 2 respectively; p = not significant; Fig. S2)

```
. ttest logFCB, by(group) unequal
```

Two-sample t test with unequal variances

Group	Obs	Mean	Std. Err.	Std. Dev.	[95% Conf.	Interval]
1	3	-1.260332	.1874178	.3246172	-2.066726	-.4539377
2	3	-.8974407	.3438403	.5955488	-2.376866	.5819846
combined	6	-1.078886	.193015	.4727884	-1.575047	-.5827252
diff		-.3628909	.3916013		-1.588419	.8626372

```
    diff = mean(1) - mean(2)                                      t =  -0.9267
Ho: diff = 0                     Satterthwaite's degrees of freedom =  3.09202

    Ha: diff < 0                 Ha: diff != 0                 Ha: diff > 0
 Pr(T < t) = 0.2103         Pr(|T| > |t|) = 0.4206          Pr(T > t) = 0.7897
```

V1/S vs. V1/S^{ctrl} (groups 1 vs. 2 respectively; p = not significant; Fig. S2)

```
. ttest logV1S, by(group) unequal
```

Two-sample t test with unequal variances

Group	Obs	Mean	Std. Err.	Std. Dev.	[95% Conf.	Interval]
1	3	-1.208234	.3612046	.6256247	-2.762372	.3459035
2	3	-1.735915	.1444376	.2501733	-2.35738	-1.11445
combined	6	-1.472075	.2102107	.514909	-2.012439	-.931711
diff		.5276807	.3890128		-.8171156	1.872477

```
    diff = mean(1) - mean(2)                                      t =   1.3565
Ho: diff = 0                     Satterthwaite's degrees of freedom =  2.62366

    Ha: diff < 0                 Ha: diff != 0                 Ha: diff > 0
 Pr(T < t) = 0.8601         Pr(|T| > |t|) = 0.2799          Pr(T > t) = 0.1399
```

F32-ART vs. F32-ART^{ctrl} (groups 1 vs. 2 respectively; p = not significant; Fig. S2)

```
. ttest logF32ART, by(group) unequal
```

Two-sample t test with unequal variances

Group	Obs	Mean	Std. Err.	Std. Dev.	[95% Conf.	Interval]
1	3	1.54359	.2493203	.4318355	.470851	2.616329
2	5	1.056817	.2388532	.5340919	.3936547	1.71998
combined	8	1.239357	.1869997	.5289149	.7971732	1.681541
diff		.4867725	.3452701		-.3917757	1.365321

```
    diff = mean(1) - mean(2)                                      t =   1.4098
Ho: diff = 0                     Satterthwaite's degrees of freedom =  5.17592

    Ha: diff < 0                 Ha: diff != 0                 Ha: diff > 0
 Pr(T < t) = 0.8921         Pr(|T| > |t|) = 0.2158          Pr(T > t) = 0.1079
```

Dd2 comparison between IPC and CNRS (groups 1 vs. 2 respectively; p = not significant; Fig. S3)

```
. ttest logDd2, by(group) unequal
```

Two-sample t test with unequal variances

Group	Obs	Mean	Std. Err.	Std. Dev.	[95% Conf.	Interval]
1	3	-1.304008	.3314896	.5741568	-2.730292	.1222767
2	2	-.4315231	.5493061	.7768362	-7.411119	6.548073
combined	5	-.9550139	.3298683	.7376079	-1.870875	-.0391527
diff		-.8724846	.6415782		-4.057465	2.312496

```
    diff = mean(1) - mean(2)                                      t =  -1.3599
Ho: diff = 0                     Satterthwaite's degrees of freedom =  1.74524

    Ha: diff < 0                 Ha: diff != 0                 Ha: diff > 0
 Pr(T < t) = 0.1615         Pr(|T| > |t|) = 0.3230          Pr(T > t) = 0.8385
```

FCB^{ctrl} comparison between NIH and CNRS (groups 1 vs. 2 respectively; p = not significant; Fig. S3)

```
. ttest logFCB, by(group) unequal
```

Two-sample t test with unequal variances

Group	Obs	Mean	Std. Err.	Std. Dev.	[95% Conf.	Interval]
1	3	-.8974407	.3438403	.5955488	-2.376866	.5819846
2	3	-1.260332	.1874178	.3246172	-2.066726	-.4539377
combined	6	-1.078886	.193015	.4727884	-1.575047	-.5827252
diff		.3628909	.3916013		-.8626372	1.588419

```
    diff = mean(1) - mean(2)                                      t =   0.9267
Ho: diff = 0                     Satterthwaite's degrees of freedom =  3.09202

    Ha: diff < 0                 Ha: diff != 0                 Ha: diff > 0
 Pr(T < t) = 0.7897         Pr(|T| > |t|) = 0.4206          Pr(T > t) = 0.2103
```

Cam5^{I543T} comparison between NIH and IPC (groups 1 vs. 2 respectively; p = not significant; Fig. S3)

```
. ttest logCam5, by(group) unequal

Two-sample t test with unequal variances

   Group |     Obs        Mean    Std. Err.   Std. Dev.   [95% Conf. Interval]
---------+--------------------------------------------------------------------
       1 |       3    3.763479    .0753753    .1305539    3.439165    4.087793
       2 |       2    3.685584    .0181838    .0257158    3.454537    3.916632
---------+--------------------------------------------------------------------
combined |       5    3.732321    .0458427    .1025074    3.605041    3.859601
---------+--------------------------------------------------------------------
    diff |            .077895    .0775377               -.2254468    .3812368
------------------------------------------------------------------------------
    diff = mean(1) - mean(2)                                      t =   1.0046
Ho: diff = 0                     Satterthwaite's degrees of freedom =   2.2245

    Ha: diff < 0                 Ha: diff != 0                 Ha: diff > 0
 Pr(T < t) = 0.7942         Pr(|T| > |t|) = 0.4116          Pr(T > t) = 0.2058
```

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