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Genome editing in the human malaria parasite *Plasmodium falciparum* using the CRISPR-Cas9 system

Mehdi Ghorbal[1–3], Molly Gorman[1,2], Cameron Ross Macpherson[1,2], Rafael Miyazawa Martins[1,2], Artur Scherf[1,2] & Jose-Juan Lopez-Rubio[1–3]

Genome manipulation in the malaria parasite *Plasmodium falciparum* remains largely intractable and improved genomic tools are needed to further understand pathogenesis and drug resistance. We demonstrated the CRISPR-Cas9 system for use in *P. falciparum* by disrupting chromosomal loci and generating marker-free, single-nucleotide substitutions with high efficiency. Additionally, an artemisinin-resistant strain was generated by introducing a previously implicated polymorphism, thus illustrating the value of efficient genome editing in malaria research.

Malaria causes ~660,000 deaths per year, and *P. falciparum* is the protozoan responsible for its most severe form. The lack of effective vaccines and the parasite's ability to develop drug resistance are major barriers to malaria treatment and eradication. Classical genome manipulation in *P. falciparum* is arduous and not robust. Zinc-finger nucleases that introduce targeted DNA double-strand breaks (DSBs) have been shown to be functional in *P. falciparum*, providing a useful means to generate targeted mutations[1]. However, although reported to be highly efficient, this technique is not widely used, mainly because of the cost and laborious design process. Recently, an alternative genome-editing strategy was developed based on the clustered, regularly interspaced, short palindromic repeat (CRISPR)–CRISPR-associated protein (Cas) system (CRISPR-Cas), a prokaryotic adaptive immune mechanism against invading viruses and plasmids[2]. *In vitro* reconstitution of the type II *Streptococcus pyogenes* CRISPR-Cas9 system showed that a single guide RNA (sgRNA) can guide the Cas9 endonuclease to cause DSBs in target DNA sites[3]. The sgRNA carries the Cas9 binding domain and a customizable 20 nucleotides (hereafter referred to as the guide), matching the target-DNA site. The protospacer-adjacent motif (PAM), a sequence immediately downstream from the target region, must be present for cleavage.

DSBs generated by Cas9 (or other genome editing technologies) can be repaired by homologous recombination using donor DNA or error-prone, nonhomologous end-joining (NHEJ), which often introduces mutations at the target site. *P. falciparum* seems to be naturally deficient in canonical NHEJ and, although an alternative NHEJ has been described, homologous recombination is the only pathway observed when an homologous sequence is present[4].

To adapt the CRISPR-Cas9 system to *P. falciparum*, we generated expression vectors for Cas9, sgRNA and donor DNA (i.e., the template for homologous recombination). Engineered *S. pyogenes* endonuclease Cas9, bearing nuclear localization signals[5], was expressed under the control of plasmodial regulatory elements in the pUF1-Cas9 episome that also carries the drug-selectable marker y*dhodh*, which gives resistance to DSM1, a *P. falciparum* dihydroorotate dehydrogenase (PfDHODH) inhibitor (**Fig. 1a**). For transcription of the sgRNA, we used *P. falciparum* U6 small nuclear (sn)RNA regulatory elements, which are thought to recruit RNA polymerase III[6] (**Fig. 1a**). The sgRNA and the donor DNA template for homologous recombination repair were placed in the same plasmid, pL7 (**Fig. 1a**).

To test sgRNA:Cas9 function in *P. falciparum*, we targeted a chromosomal *egfp* locus[1]. We used an sgRNA targeting the *egfp* gene (guide^{egfp}) and a donor DNA template consisting of two homology regions flanking the drug-selectable marker-cassette h*dhfr (*human dihydrofolate reductase, which confers resistance to the antifolate drug WR99210, referred to as WR). Plasmids pL7-*egfp* and pUF1-Cas9 were co-transfected and WR selection was applied; hence, the Cas9 protein was only transiently expressed. WR-resistant parasites were obtained within 3 weeks of transfection. pL7-*egfp* also includes the suicide-gene y*fcu* (yeast cytosine deaminase and uridyl phosphoribosyl transferase) allowing 5-fluorocytosine selection to be used to kill parasites carrying copies of pL7-*egfp*. PCR analysis showed complete disruption of the *egfp* locus and integration of the WR-resistance cassette through double-crossover recombination (**Fig. 1b**). The absence of EGFP-positive cells was further confirmed by flow cytometry (**Fig. 1c**). As a control, parasites were also transfected with pUF1-Cas9 and a version of pL7-*egfp* lacking the guide sequence (called pL6-*egfp*); these had similar proportions of EGFP-positive cells to the wild-type parasites with no evidence of recombination (**Fig. 1b**,**c**).

We also tested *P. falciparum*-gene disruption using linear DNA, which has, to our knowledge, not been reported despite numerous attempts[7], but is known to be functional in the rodent malaria parasite *Plasmodium berghei*. Following the procedure described above but using linearized pL7-*egfp* (**Supplementary Fig. 1**), we obtained WR-resistant parasites with a disrupted *egfp* locus within the same time period as for circular pL7-*egfp* (**Fig. 1b**,**c**). Linear DNA is apparently lost after 4 d in *P. falciparum*[8], so the success of using linear DNA suggests efficient cleavage and repair of the target sequence during the first few growth cycles after transfection. Using linear DNA also avoids the need for negative selection against parasites retaining the plasmid and could enable cloning-free strategies to provide donor DNA[9]. Targeting the nonessential, endogenous, knob-associated, histidine-rich protein coding gene (*kahrp*)[10] using a linearized plasmid containing the sgRNA and a donor sequence consisting of two

[1]Biology of Host-Parasite Interactions Unit, Institut Pasteur, Paris, France. [2]CNRS URA 2581, Institut Pasteur, Paris, France. [3]Present address: CNRS 5290/IRD 224/University Montpellier 1&2 ("MiVEGEC"), Montpellier, France. Correspondence should be addressed to J.-J.L.-R. (jose-juan.lopez-rubio@inserm.fr).

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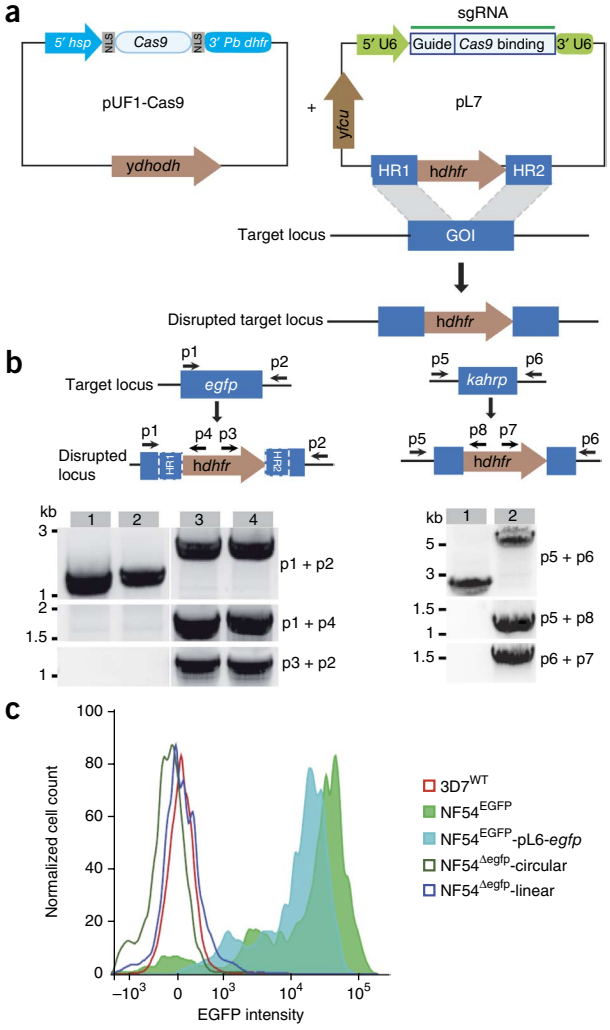

Figure 1 Targeted *P. falciparum* genome editing using sgRNA:Cas9. (**a**) The Cas9 endonuclease bearing nuclear localization signals (NLS) is expressed in the pUF1-Cas9 episome using plasmodial regulatory elements. The pL7 episome contains the sgRNA-expression cassette and donor DNA. sgRNA is expressed from the *P. falciparum* U6 snRNA polymerase III promoter (5′ U6). To knock out genes, homology region 1 (HR1) and homology region 2 (HR2) of the gene of interest (GOI) must surround a drug-selectable marker (h*dhfr*). 5′ *hsp*, heat shock protein 86 promoter region; 3′ *Pb dhfr*, 3′ region of *P. berghei dhfr*; y*dhodh*, yeast dihydroorotate dehydrogenase gene. (**b**) Left: disruption of the chromosomal *egfp* locus using sgRNA:Cas9, analyzed by PCR using the primers indicated as p1–p4. Lane 1: parental line NF54^{EGFP}. Lane 2: parental line NF54^{EGFP} stably carrying pUF1-Cas9 and pL6-*egfp* episomes. Lane 3: WR and 5-fluorocytosine–resistant NF54$^{\Delta egfp}$ parasites resulting from transfection with circular pL7-*egfp* and transient pUF1-Cas9. 5-fluorocytosine pressure was applied after WR-resistant parasites appeared; genomic DNA for PCR analysis was collected after 2 weeks. Lane 4: WR-resistant NF54$^{\Delta egfp}$ parasites resulting from transfection with linearized pL7-*egfp* and transient pUF1-Cas9. Right: disruption of the *kahrp* gene using sgRNA:Cas9 and integration of the selectable h*dhfr* cassette were analyzed by PCR (primers p5–p8). Lane 1: parental line 3D7^{WT}. Lane 2: mutant line 3D7$^{\Delta kharp}$. (**c**) Flow-cytometry analysis of 3D7^{WT} (open red), NF54^{EGFP} (solid green), NF54^{EGFP}-pL6-*egfp* (solid blue), NF54$^{\Delta egfp}$ (open green and open blue). WT: wild type.

sections of the *kahrp*-coding sequence flanking the h*dhfr* cassette also yielded recombinant WR-resistant parasites and *kahrp*-gene disruption on a time scale similar to that for *egfp* (**Fig. 1b**). We also tested a recently described transfection method[11] reported to improve efficiency and detected WR-resistant parasites bearing the disrupted *egfp* locus as early as day 8 post-transfection (data not shown).

To test introduction of a single point mutation without integrating a selectable marker, we designed a donor DNA carrying the desired mutation and an additional modification (defined here as shield mutation) at the Cas9-target site. The shield mutation is silent but abolishes recognition by Cas9, thereby protecting the modified locus from repeated cleavage[1] (**Fig. 2a** and **Supplementary Fig. 1**). Notably, the distance between the shield and desired mutations should be minimized to reduce the possibility of only the shield modification being incorporated.

First, we generated plasmid pL7-*orc1* bearing an sgRNA targeting the *orc1* gene and a donor DNA of ~900 bp containing a mutation to substitute Orc1 leucine 137 with alanine (**Fig. 2a**,**b**). This mutation has previously been shown to prevent oligomerization of recombinant Orc1, leading to the loss of telomeric-DNA binding *in vitro*[12]. It is predicted to affect mono-allelic expression of the subtelomeric multigene *var* gene family, encoding a major *P. falciparum* virulence factor. Within 3 weeks of transfection, parasites carrying both pL7-*orc1* and pUF1-Cas9 were obtained by WR and DSM1 selection. Sanger and whole-genome sequencing confirmed the presence of the desired mutations at the endogenous locus and no evidence of remaining copies of the wild-type locus (**Fig. 2b**). Despite predictions, *var* gene-expression profile analysis of three mutant clones did not show clear *var* gene deregulation (**Supplementary Fig. 2a**). Also, immunofluorescence analysis with anti-Orc1 antibodies showed that nuclear localization was not disrupted, unlike in the previous work[12] (**Supplementary Fig. 2b**).

Recently, a correlation has been described between resistance to artemisinin derivatives and a mutation (C580Y) in the *P. falciparum* PF3D7_1343700 kelch propeller domain (K13-propeller)[13]. To validate the candidate drug-resistance locus, we introduced this mutation using sgRNA:Cas9 (**Fig. 2c** and Online Methods). We obtained independent clones by limiting dilution and used Sanger and whole-genome sequencing to confirm the presence of the desired modifications (**Fig. 2c**). To assess whether the introduced mutation is associated with an artemisinin-resistance phenotype, we tested ring-stage parasite survival ($RSA_{0-3\,h}$ survival assay) and found that the C580Y-mutation is associated with increased survival (~13.5%; **Fig. 2d**), similar to the rate previously reported for Cambodian parasite isolates carrying the mutation (14.1%)[13]. This confirms the contribution of K13-propeller C580Y polymorphism to artemisinin resistance.

To assess possible off-target mutations, we followed a two-step approach. First, we scanned the *P. falciparum* reference genome (Pf3D7_v3) for potential off-target sites by comparing the guide sequence to genomic regions flanking PAMs (NGG and NAG). We then performed whole-genome sequencing of parental and mutant lines and searched for PAM-associated indels uniquely present in mutant lines. We found no evidence to suggest off-target activity (**Supplementary Results**, **Supplementary Table 1** and **Supplementary Fig. 3**); this is not surprising because the *P. falciparum* end-joining pathway is highly inefficient and the vast majority of cells with off-target DNA cleavage would not survive[4].

When we used an NGG PAM, we found the *P. falciparum* genome contained 663,952 possible target sites spaced at an average distance of 35 bp, meaning that virtually any *P. falciparum* locus could be targeted by CRISPR-Cas9 (**Supplementary Fig. 4**). In other systems, a guanosine is preferred at the sgRNA 5′ position for efficient RNA polymerase III transcription[14], but for the sgRNAs we tested this was not required (**Supplementary Table 2**). For other organisms, reported CRISPR-Cas9 genome editing efficiencies differed depending on the target site[14]. In our study, all of the target sites were edited without redesign of any guide sequences and so, because of that and the frequency of the NGG motif, we suggest that sgRNA design should be straightforward for *P. falciparum*.

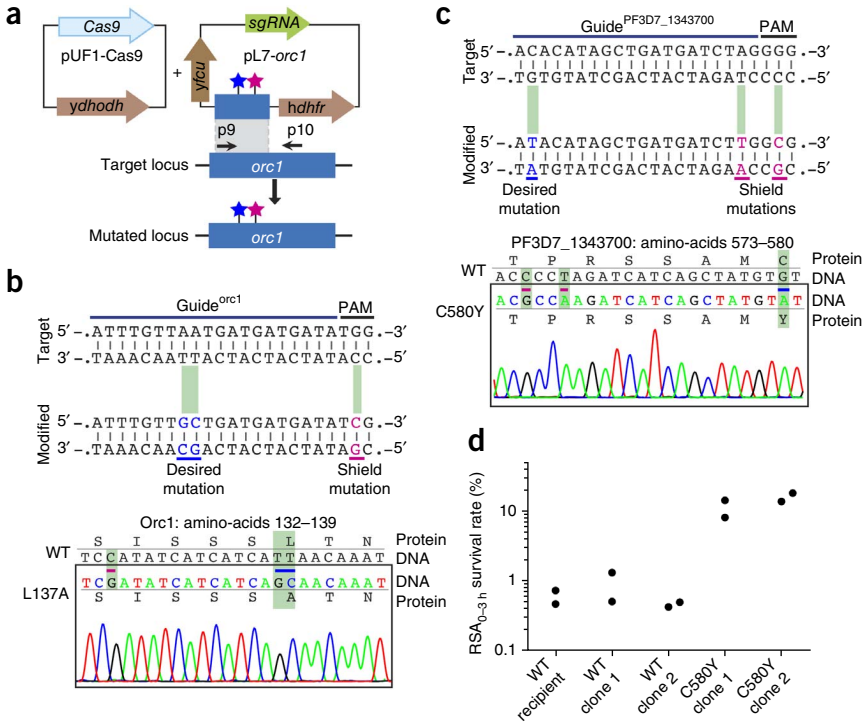

Figure 2 Marker-free nucleotide editing using sgRNA:Cas9 in *P. falciparum*. (**a**) Diagram illustrating the strategy used for marker-free nucleotide replacement, in this case for the *orc1* gene. The Cas9 protein is expressed in the pUF1-Cas9 episome continuously maintained using the y*dhodh* drug-selectable marker. pL7-*orc1* episome is continuously maintained using the h*dhfr* selection and carries both the sgRNA^{orc1} and the donor DNA (blue box). The donor DNA carries the designed desired mutation (blue star) and the shield mutation (magenta star). (**b**) Top: target sequence recognized by sgRNA^{orc1}:Cas9 highlighting the 20-nucleotide guide sequence and the PAM (top). Modified locus showing the desired mutations (blue) to generate the replacement L137A and shield mutation (magenta) (bottom). The latter is essential to prevent cleavage of the modified locus by Cas9. To obtain the Orc1 modification L137A, the desired mutation may fulfill the shield role because it is included in the guide sequence; however, we included an additional shield modification to make it more robust. Bottom: chromatograms showing 3D7^{WT} and 3D7$^{orc1\text{-}L137A}$ sequence analyses. The designed nucleotide mutations and the amino-acid change are highlighted. (**c**) Top: target sequence recognized by sgRNA^{PF3D7_1343700}:Cas9 highlighting the 20-nucleotide guide sequence and the PAM (top). Modified locus showing the desired mutations (blue) to generate the replacement C580Y and shield mutations (magenta) (bottom). Bottom: chromatograms showing sequence analyses and corresponding amino acids. The designed nucleotide mutations and the amino-acid change are highlighted. (**d**) Marker-free nucleotide editing of the PF3D7_1343700-C580Y kelch propeller domain mutation associated with artemisinin resistance[13]. Survival rates of wild-type NF54 recipient line and wild-type clones and PF3D7_1343700-C580Y mutant clones in the $RSA_{0-3\,h}$ assay. Rates are plotted on a log scale. Mutant clones have higher $RSA_{0-3\,h}$ rates than wild-type parasites: wild-type means (0.61%, 0.93% and 0.47%); PF3D7_1343700-C580Y means (11.14% and 15.81%). $n = 2$.

To our knowledge, this demonstration of CRISPR-Cas9 genome editing in *P. falciparum* is the first in a eukaryotic pathogen. We showed that specific gene knockouts and single-nucleotide substitutions can be achieved in a short time frame, in contrast to the 2–4 months (occasionally >18 months[1]) required using conventional methods. Also, only a limited number of selectable markers are available for *P. falciparum*, so the success of this marker-free approach is crucial for consecutive genome manipulations.

The CRISPR-Cas9 system in *P. falciparum* opens opportunities for various applications. For example, the lack of tools for targeted gene regulation in *P. falciparum* could be overcome through the use of catalytically inactive Cas9 fused to repressive or activating effectors, as shown for other organisms[15]. We foresee CRISPR-Cas9 becoming a routine laboratory technique in malaria research and perhaps for other medically and economically important pathogens.

METHODS

Methods and any associated references are available in the online version of the paper.

Accession codes. NCBI BioProject: PRJNA246313.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

J.-J.L.-R. devised the research experimental design with contributions from Me.G. Mo.G., Me.G. and J.-J.L.-R. performed the experiments. R.M.M. prepared the libraries for next-generation sequencing. C.R.M. conducted the bioinformatic analysis. A.S. provided funding. J.-J.L.-R. and Me.G. wrote the manuscript with contributions from all the authors.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Plasmid constructs. The Cas9-expressing construct (pUF1-Cas9) was generated by amplification of the Cas9-coding sequence from the plasmid pX330 (Addgene plasmid 42230 (ref. 5)) using primers P11 and P12, and its cloning into the pUF1 plasmid[16] by replacing the *yfcu*-coding sequence using restriction sites XhoI and SmaI.

The pL6 plasmid, containing the sgRNA-expression cassette and the homology regions, was constructed in multiple cloning steps. The sgRNA-expression cassette was constructed by amplification and cloning of the U6-regulatory elements using primers P17/P18 and P19/P20 and restriction sites NcoI and BstAPI, the pre-sgRNA containing the trackRNA and the BtgZI-adaptor was ordered as a DNA block from Integrated DNA Technologies (gRNA, see **Supplementary Table 2**). The design was based on the previously described sgRNA[17]. To generate the pL6-*egfp*, we amplified the *egfp* homology regions using primers P13/P14 and P15/P16 from genomic DNA NF54^{EGFP} and cloned them using the restriction sites AflII/SpeI and NcoI/EcoRI. To generate the pL6-*kahrp* plasmid, we amplified the *kahrp* homology regions using primers P21/P22 and P23/P24 from 3D7 genomic DNA, and cloned them using the restriction sites AflII/SpeI and NcoI/EcoRI. To generate pL6-*orc1*, we amplified two fragments of *orc1*-gene-coding sequence using primers P25/P26 and P27/P28 bearing the desired mutations and the 15-bp homology necessary for InFusion cloning. To generate pL6-*PF3D7_1343700*, we amplified two fragments of *PF3D7_1343700* coding sequence using primers P37/P38 and P39/P40 bearing the desired mutations and the 15-bp homology necessary for InFusion cloning. Cloning used the restriction sites AflII and SpeI. All PCR amplifications were done with high-fidelity polymerase PfuUltra II Fusion HS DNA polymerase (Agilent Technologies) following the recommended protocols, except we lowered the elongation temperature to 62 °C or 68 °C. All cloning reactions used the In-Fusion HD Cloning Kit (Clontech) and followed the manufacturer's protocol. All cloning and plasmid amplifications were done in *Escherichia coli*, XL10-Gold Ultracompetent Cells (Stratagene).

For all the targets, the pL7 constructs were made by replacement of the BtgZI-adaptor with the guide DNA sequence (**Supplementary Fig. 1c**). For each cloning, the corresponding pL6 plasmid bearing the homology regions was digested using restriction enzyme BtgZI for 2 h at 60 °C then purified with PCR Clean Up Kit (Macherey-Nagel) and subsequently in agarose gel using the same kit. Guide-RNA cloning used the In-Fusion HD Cloning Kit, with the 20-bp guide RNA surrounded by the 15 bp necessary for InFusion cloning, ordered as two oligonucleotides and annealed. The oligonucleotides used were P29/P30, P31/P32, P33/P34 and P35/P36 for *egfp*, *kahrp*, *orc1* and *PF3D7_1343700* constructs, respectively (**Supplementary Table 2**). Sanger sequencing confirmed the absence of undesired mutations in the homology regions and the guide sequence.

Parasite culture and transfections. Asexual blood-stage parasites (NF54 and 3D7 wild-type strains and their derived mutants) were cultured in human red blood cells (RBCs) in RPMI-1640 culture medium supplemented with Albumax (Gibco Life Technologies), hypoxanthine (C.C.Pro GmbH) and gentamicin (Sigma) at 5% hematocrit under 5% O_2/3% CO_2 at 37 °C. Synchronous cultures were obtained by sorbitol treatment and/or plasmion enrichment.

Parasites were transfected as previously described either by electroporating ring-stage parasites[18] or nucleofection of schizont stages[11,19]. For transfections with circular DNA, 50 µg of each plasmid were used. For transfections with linear DNA, pL7-*egfp* or pL7-*kahrp* plasmid was linearized with double-cutting restriction enzyme HincII, extracted by phenol-chloroform, verified for linearization in agarose gel and 10 µg were subsequently used for transfection. Prior to transfection, the appropriate DNA was ethanol-precipitated and resuspended in 10 µl and 30 µl of Tris-EDTA buffer for schizont nucleofection and ring-electroporation, respectively.

Drug pressure was applied 15–20 h after transfection and media and drugs were renewed every 24 h for the first 5 d. WR99210 (Sigma-Aldrich) was used at 2.66 nM. When continuous Cas9-protein expression was required, DSM1 (ref. 16) was applied at 1.5 µM. Negative selection was applied with 5-fluorocytosine 40 µM[7].

Analysis of mutant parasite strains. In all mutants, target-gene disruption and h*dhfr*-cassette integration were verified by PCR using *Taq* polymerase (MPBio), according to the manufacturer's instructions. Genomic DNA was extracted from infected RBCs using the DNeasy Blood & Tissue Kit (Qiagen) following the nucleated-erythrocyte protocol described therein.

3D7$^{ORC1\text{-}L137A}$ and NF54$^{PF3D7_1343700\text{-}C580Y}$ mutant parasites were analyzed after amplification of the locus using the high-fidelity polymerase PfuUltra II Fusion HS DNA polymerase and the primers P9/P10 and P41/P42 respectively. The PCR product was then purified using the PCR CleanUp Kit and sequenced using the same primers.

Whole-genome sequencing and data processing. Indexed DNA libraries for next-generation sequencing (NGS) were prepared using the TruSeq Nano DNA LT Sample Prep Kit (Illumina), with minor modifications. Genomic DNA was purified from 3D7^{WT} bulk recipient strain, 3D7$^{ORC1\text{-}L137A}$ and 3D7$^{\Delta KAHRP}$ mutant bulk culture parasites, NF54^{WT} bulk recipient strain and NF54$^{PF3D7_1343700\text{-}C580Y}$ mutant clones (NF54$^{PF3D7_1343700\text{-}C580Y_C1}$ and NF54$^{PF3D7_1343700\text{-}C580Y_C2}$) with DNeasy Blood and Tissue Kit. Genomic DNA (1.25 µg) in 125 µl of Tris-EDTA buffer was fragmented by high-power sonication in a Bioruptor UCD-200 (Diagenode), for three 10-min cycles, with 30 s ON pulses, followed by 30 s OFF. DNA fragments (100 ng) were used for library preparation following the manufacturer's instructions, except for the elongation temperature in the final PCR-amplification step, lowered to 68 °C. After purification, fragment sizes and library quality were estimated in a Bioanalyzer 2100 (Agilent) and the DNA concentration was determined in a Qubit Fluorometer (Life Technologies). Then, Genoscreen sequenced 100-bp reads in a MiSeq sequencer (Illumina).

Single-end 100-nt reads from each fastq-sample file (3D7$^{WT,ORC1\text{-}L137A,\Delta KAHRP}$ and NF54^{WT} populations, and NF54$^{PF3D7_1343700\text{-}C580Y_C1,\ PF3D7_1343700\text{-}C580Y_C2}$ clones) were mapped to Pf3D7_v3 using the BWA-MEM algorithm found in BWA v0.7.5a and processed using SAMtools v0.1.18. In all 3D7 samples, > 99% of reads were mapped to the genome with an average 34× depth of coverage. Moreover, reads from all samples covered an average of 98.85% of the reference genome by at least five reads. For NF54 samples, an average of 80% of the reads mapped at 20× depth of coverage; and, at least 95.58% of the reference genome was represented by at least five reads in each sample.

For samples from the 3D7$^{\Delta KAHRP}$ line, the clipped portions of partially mapped reads were extracted using custom-made scripts and the pysam module v0.7.5 for Python v2.7. These clipped reads were then mapped to the relevant plasmid sequence, which resulted in clusters of clipped reads and indicated regions of possible recombination. Tracing these clusters back to their original locations on the Pf3D7_v3 genome helped identify true recombination sites. Moreover, all reads were mapped to the relevant plasmid sequence to compare plasmid versus Pf3D7_v3 depth of coverage.

VarScan2 was used to detect SNPs in the NF54$^{WT,\ PF3D7_1343700\text{-}C580Y_C1,\ PF3D7_1343700\text{-}C580Y_C2}$ and 3D7$^{WT,ORC1\text{-}L137A}$ samples. Here, we found that the 3D7$^{ORC1\text{-}L137A}$ line and both of the NF54 clones contained their intended CRISPR-Cas9 mutations (present in 100% of representative reads); whereas, their respective parental lines were absent of any such evidence.

Flow cytometry analysis of recombinant parasites. The proportions of EGFP-positive parasites in the NF54^{EGFP} parental strain and the Cas-mutated strains were determined by flow-cytometry analysis. Resuspended cultures (10 µl) were stained for 15 min with 4 µM-PBS Hoechst 33342 DNA stain (Sigma). Cells were analyzed using a BD LSRFortessa cell analyzer and 30,000 events were recorded. Collected data were analyzed with FlowJo vX.0.6.

***var* gene-expression analysis by quantitative PCR (qPCR).** RNA was extracted from ring-stage wild-type and mutant strain cultures with 10 volumes (cell pellet) of Trizol (Life Technologies) following the manufacturer's instructions. DNA contamination was removed by RNase-free DNase (Ambion). cDNA was synthesized by random primer mix using Superscript III Reverse Transcriptase (Life Technologies) according to the product manual. PCR primers to detect *var* genes mRNA expression were used as described previously[20]. A housekeeping gene, seryl tRNA synthetase (PF3D7_0717700), served to normalize transcription levels of each *var* gene.

Immunofluorescence microscopy. Synchronized *P. falciparum* cultures were washed in phosphate-buffered saline (PBS), lysed in saponin (0.15%)

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and fixed in suspension with 4% paraformaldehyde-PBS solution for 60 min at room temperature. Parasites were then washed in PBS and incubated with the primary anti-Orc1 (ref. 21) antibody, diluted in 1% bovine serum albumin (BSA–PBS) at 37 °C for 30 min. After washing, parasites were incubated at 37 °C for 30 min with the secondary goat anti-rabbit-Alexa 488-nm antibody (Invitrogen), diluted in 1% BSA-PBS. After final washes, parasites were deposited on microscope slides and mounted in Vectashield anti-fading with 4-6-diamidino-2-phenylindole (DAPI). Images were captured using a Nikon Eclipse 80i optical microscope. Anti-Orc1 and goat anti-rabbit-Alexa 488-nm antibodies were pre-adsorbed with lysed noninfected RBCs before incubation with the fixed parasites. The final antibody dilutions were: rabbit anti-Orc1 1:100, Alexa-Fluor-488-conjugated goat anti-rabbit 1:2,000.

Ring-stage survival assay. The ring-stage assay ($RSA_{0\text{-}3\ h}$) was done as previously described[22]. Briefly, highly synchronous parasites cultures at 0–3 h post-invasion ring-stage parasites were exposed to 700 nM dihydroartemisinin or its solvent DMSO for 6 h, washed and then cultivated for the next 66 h without drug. Survival rates were assessed microscopically by counting in Giemsa-stained smears the proportion of viable parasites that developed into second generation rings or trophozoites.

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