Efficient CRISPR-Cas9–mediated genome editing in *Plasmodium falciparum*

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Malaria is a major cause of global morbidity and mortality, and new strategies for treating and preventing this disease are needed. Here we show that the *Streptococcus pyogenes* Cas9 DNA endonuclease and single guide RNAs (sgRNAs) produced using T7 RNA polymerase (T7 RNAP) efficiently edit the *Plasmodium falciparum* genome. Targeting the genes encoding native knob-associated histidine-rich protein (*kahrp*) and erythrocyte binding antigen 175 (*eba-175*), we achieved high (≥50–100%) gene disruption frequencies within the usual time frame for generating transgenic parasites.

The most commonly used approach for modifying chromosomal loci in *P. falciparum* relies on spontaneous single- or double-crossover recombination using plasmids containing sequence homologous to the target region[1,2]. This is extremely inefficient and time consuming. Circular plasmids are used to transform *P. falciparum*, and these are preferentially maintained as episomes. Isolating rare parasites with the desired chromosomal integration event from a high episomally transformed background requires protracted on-off selection drug cycling and/or negative-selection procedures, none of which increases the initial fraction of the population genetically modified as desired. Zinc-finger nucleases have recently been used to efficiently achieve targeted knockouts and allele replacements in *P. falciparum*[3], but these nucleases take significant effort to make, and efficiency is not guaranteed[4,5]. Recently, efficient site-specific genome editing using Cas9 has been shown in several organisms[6–10], including in *P. falciparum*[11] while this work was under review. Here we apply the clustered, regularly interspaced, short palindromic repeats (CRISPR)-Cas9 system for genome engineering, using a *T7* promoter to express sgRNA and target two endogenous loci in *P. falciparum*.

First we implemented a strategy for producing sgRNAs *in situ*. Although the strong *U6* promoter transcribed by the endogenous polymerase Pol III has been used to produce functional sgRNAs in other organisms[7,8,10], this promoter has not been well defined in *P. falciparum*. Several *P. falciparum* Pol II promoters have been described. However, target transcripts produced from these promoters have long, heterogeneously sized 5′ and 3′ flanking regions[12,13] and are likely produced at lower levels than Pol III-synthesized transcripts. Given this, we chose T7 RNA polymerase (T7 RNAP) for producing defined sgRNAs in *P. falciparum*, as it uses well-characterized promoter and terminator sequences to make transcripts of defined size and in high yield.

We first validated that functional T7 RNAP could be expressed in *P. falciparum*. We constructed a vector to express T7 RNAP (pT7 RNAP) using pfGNr (MRA-462; http://www.mr4.org/) as a starting point (**Fig. 1a**). pT7 RNAP and pfGNr were separately transfected into 3D7 parasites, and G418-selected episomal lines were obtained. Western blot analysis confirmed that intact T7 RNAP was produced in the pT7 RNAP– but not pfGNr–transfected lines (**Fig. 1b**). To establish that the T7 RNAP produced was enzymatically active, we constructed several additional vectors (**Fig. 1a**) and transfected 3D7 parasites with various combinations of these (**Fig. 1c,d**). On the basis of quantitative reverse-transcription PCR (RT-PCR) and northern blot analyses, T7 RNAP made in the parasite was enzymatically active and, expectedly, produced target transcripts of the expected size only when a *T7* promoter was present. Notably, T7 RNAP expression in either the absence or presence of a *T7* promoter–driven expression cassette was well tolerated. No gross effects on parasite viability in the short term (**Fig. 1e**) or after prolonged periods of continuous culture (data not shown) were observed.

Guide RNAs produced *in vitro* using T7 RNAP and linearized plasmid DNA templates have been used to successfully induce Cas9-mediated editing in zebrafish and fruit flies[6,9]. Because *P. falciparum* maintains circular plasmids, a *T7* terminator, which will be transcribed, is required to specify transcription termination. This adds a stem loop at the 3′ end of the sgRNA (sgRNA-T) (**Supplementary Fig. 1**). We confirmed that sgRNA-T can direct specific cleavage of a DNA template *in vitro*. We *in vitro*–transcribed test *kahrp*-sgRNA-T and *eba-175*–sgRNA-T and a control pUC19-sgRNA-T from circular plasmid templates. We then co-incubated Cas9-containing cell lysates, DNA templates containing sgRNA target sites and either *kahrp*-sgRNA-T and *eba-175*–sgRNA-T or control (pUC19-sgRNA-T) to test cleavage activity. Both the *kahrp*- and *eba-175*–sgRNA-Ts specifically and efficiently cleaved their respective target DNA, whereas the pUC19-sgRNA-T did not (**Supplementary Fig. 1**). Thus, the additional sequence introduced when using the *T7* terminator did not interfere with Cas9 and sgRNA-T–mediated target cleavage *in vitro*.

Next we determined whether sgRNA-T produced *in situ* could be used to successfully disrupt chromosomal loci in *P. falciparum*.

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RECEIVED 3 MARCH; ACCEPTED 8 JULY; PUBLISHED ONLINE 10 AUGUST 2014; DOI:10.1038/NMETH.3063

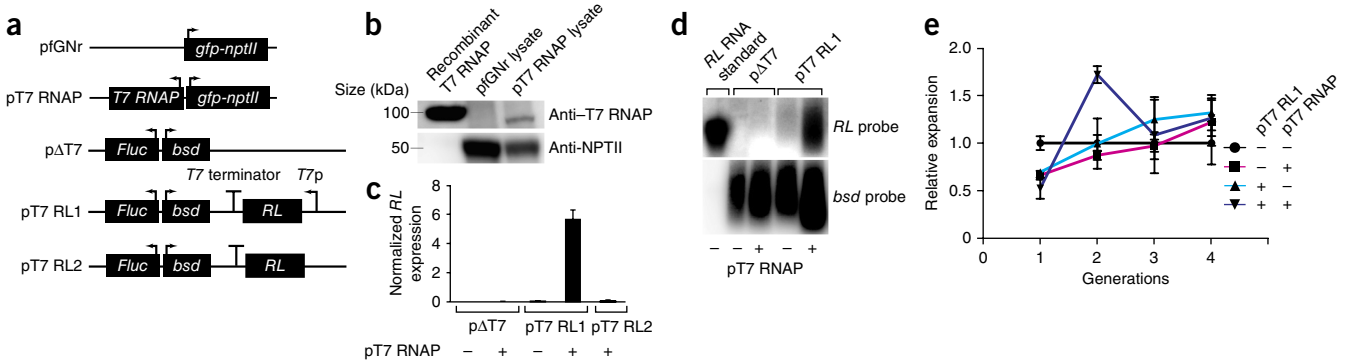

Figure 1 | Validating T7 RNAP functions in *P. falciparum*. (**a**) Plasmids used to test T7 RNAP expression and activity. *bsd*, blasticidin-S deaminase; *Fluc*, firefly luciferase; *gfp-nptII*, green fluorescent protein–neomycin phosphoribosyl transferase II; *RL*, *Renilla* luciferase. (**b**) Western blot analysis of T7 RNAP protein production in parasites. Blots were probed with anti–T7 RNAP and anti-NPTII (loading control) antibodies. (**c**) Quantitative RT-PCR analysis of normalized *RL* transcript levels produced after the indicated transfections. Data are mean ± s.d. (n = 3 technical replicates). (**d**) Northern blot analysis for *RL* transcript production, with the *bsd* selection marker transcript probed as a control. (**e**) Relative parasite growth over four successive generations in the presence or absence of T7 RNAP and a *T7* promoter–driven expression cassette. Data are mean ± s.d. (n = 3 biological replicates) analyzed for significance by one-way ANOVA (P = 0.78).

We selected the *kahrp* (PlasmoDB ID: PF3D7_0202000) locus on *P. falciparum* chromosome 2 as an initial target. This gene is involved in the formation of knobby projections on the surface of *P. falciparum*–infected red blood cells, and its disruption produces infected red blood cells that have a smooth surface phenotype[1]. For experimental ease and generalizability with which new loci can be targeted, we constructed two base plasmids (**Fig. 2a**). The first (pCas9-sgRNA-T) delivers Cas9 and the sgRNA-T targeting a specified locus. The second (pT7 RNAP-HR) delivers T7 RNAP and encodes a homologous region to repair the Cas9–sgRNA-T–induced DNA double-strand break. We designed the homologous region such that successful chromosomal editing results in an in-frame transcriptional fusion of a T2a peptide–*Renilla* luciferase (T2a-*RL*) coding sequence[14] with the upstream target gene fragment. A stop codon is included at the end of the *Renilla* gene to terminate translation. Thus, successful editing is expected to result in *Renilla* luciferase expression if the targeted gene's promoter is transcriptionally active. We initially created three versions of pCas9-sgRNA-T from which either no sgRNA (pCas9-No sgRNA-T), pUC19-sgRNA-T (pCas9-pUC19 sgRNA-T) or *kahrp*-sgRNA-T (pCas9-*kahrp* sgRNA-T) is expressed. We separately cotransfected these plasmids with pT7 RNAP-HR^{kahrp} and continuously selected for episomal retention of both over the typical period (~4–6 weeks) required for obtaining easily manipulated cultures (≥1% parasitemia (the fraction of parasite-infected red blood cells)). We used either a 3D7^{attB} parasite line with a reference firefly luciferase gene site-specifically integrated at the *cg6* locus[14] or a parental 3D7 line (knob positive) in our experiments.

We monitored *Renilla* and firefly luciferase levels periodically during the course of transfection as a simple readout of successful editing within the parasite population. On post-transfection day 33, we detected a substantial increase in relative *Renilla*-to-firefly luciferase signal for parasites transfected with *kahrp*-sgRNA-T but not the control pUC19-sgRNA-T (**Fig. 2b**). We used PCR to analyze isolated genomic DNA to determine whether the *kahrp* gene had been disrupted by insertion of the T2a-*RL* cassette in the parasite population expressing *kahrp*-sgRNA-T but not the pUC19-sgRNA-T. Diagnostic PCR yielded products only for *kahrp*-sgRNA-T and not pUC19-sgRNA-T transfections (**Fig. 2c**). Next we performed Southern blot analysis to confirm that the targeted locus had been modified as expected and to estimate the frequency of editing within the parasite population. These data indicated that virtually the entire parasite population transfected with *kahrp*-sgRNA-T and pT7 RNAP-HR^{kahrp} had been successfully edited, whereas in pUC19-sgRNA-T– and pT7 RNAP-HR^{kahrp}–transfected parasites, the native *kahrp* locus remained intact (**Fig. 2d**). Consistent with these findings, we were unable to detect KAHRP protein expression by western blot analysis of the *kahrp*-sgRNA-T–transfected parasite population. In contrast, we readily detected KAHRP in the pUC19-sgRNA-T control parasites (**Fig. 2e**).

In a parallel and independent experiment, we examined editing induced by the *kahrp*-sgRNA-T versus a No sgRNA-T control in a parental 3D7 strain. Beginning on day 20, *Renilla* luciferase levels steadily increased above background and became elevated in parasites transfected with *kahrp*-sgRNA-T but not in the No sgRNA-T control (**Supplementary Fig. 2a**). As before, PCR analysis revealed editing at the *kahrp* locus only in the *kahrp*-sgRNA-T transfection, and western blot confirmed that virtually no KAHRP protein was expressed at the population level (**Supplementary Fig. 2b**). Additionally, using scanning electron microscopy (SEM), we confirmed that parasites transfected with the no-sgRNA control plasmid retained the 'knobby' phenotype associated with an intact *kahrp* gene. However, the *kahrp*-sgRNA-T–transfected parasites appeared smooth, as expected for *kahrp*-null parasites (**Fig. 2f**). Finally, we demonstrated disruption of the *kahrp* gene in four clones obtained by limiting dilution from the *kahrp*-sgRNA-T–transfected parasite pool by PCR and western blot (**Fig. 2g,h**).

Next we examined Cas9-mediated editing at a second, unrelated genomic locus. We selected the *eba-175* gene (PlasmoDB ID: PF3D7_0731500) on chromosome 7, which encodes a parasite ligand used during invasion of red blood cells[15]. We constructed *eba-175*–sgRNA-T expression (pCas9-eba-175 sgRNA-T) and donor (pT7 RNAP-HR$^{eba\text{-}175}$) plasmids (**Supplementary Fig. 3a**) and transfected these as described before. To evaluate editing, we isolated genomic DNA from parasites transfected in

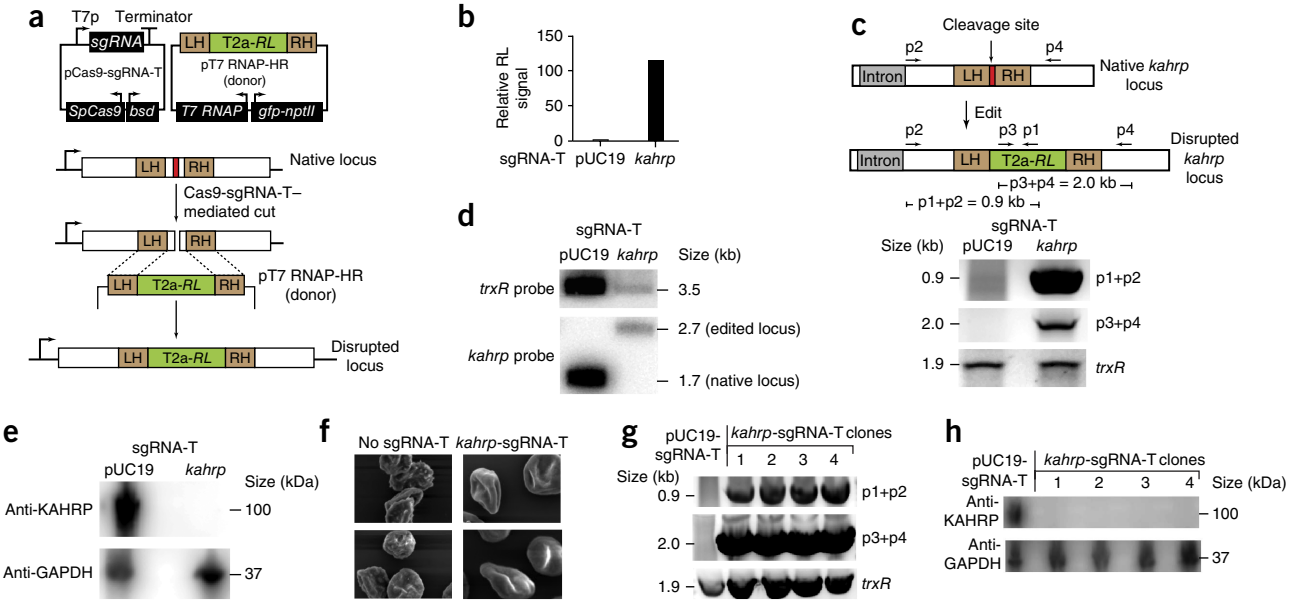

Figure 2 | CRISPR-Cas9–mediated disruption of the *P. falciparum kahrp* locus. (**a**) Generalized schematic of the Cas9 and *T7* promoter–driven sgRNA-T plasmid used for genome editing. For homology-directed repair of the induced double-strand break, pT7 RNAP is modified to include left homologous (LH) and right homologous (RH) regions flanking a T2a-*RL* gene. (**b**) Measurement of RL expression in a parasite population when the *kahrp* locus is targeted by *kahrp*-sgRNA-T or the pUC19-sgRNA-T control in the presence of a suitable donor plasmid. (**c**) PCR primers (p1, p2, etc.) to specifically detect homology-directed repair at a target cut site in the *kahrp* locus amplify products of the expected size for *kahrp*-sgRNA-T– but not pUC19-sgRNA-T–transfected parasite populations. (**d**,**e**) Southern (**d**) and western blot analyses (**e**) of parasite populations obtained after transfection with the pUC19- and *kahrp*-sgRNA-Ts. trxR, thioredoxin reductase, is used as a positive control. (**f**) SEM imaging analysis of parasite populations obtained after transfection with a No sgRNA-T control and *kahrp*-sgRNA-T. (**g**,**h**) PCR and western blot analyses as in (**c**) and (**e**), respectively, of cloned parasites derived from the *kahrp*-sgRNA-T edited pool. The unedited pUC19-sgRNA-T pool with an intact native *kahrp* locus is used as a positive control.

parallel with pCas9–*eba-175* sgRNA-T and pT7 RNAP-HR$^{eba\text{-}175}$ (test) and pCas9-pUC19 sgRNA-T and pT7 RNAP-HR$^{eba\text{-}175}$ (control) at ~5 weeks post transfection. Using PCR and sequencing of the resulting products, we determined that the expected editing events upstream and downstream of the induced cut site had both occurred in the *eba-175*–sgRNA-T–expressing but not the control pUC19-sgRNA-T–expressing parasite population (**Supplementary Fig. 3b,c**). Through Southern blot analysis, we again confirmed that the targeted locus was modified as expected (**Supplementary Fig. 3d**). These data suggest that ~50% of parasites within the population had been successfully edited. To address reproducibility and consistency, we repeated transfections targeting the *eba-175* locus in biological triplicates. We again verified that only pCas9–*eba-175* sgRNA-T and pT7 RNAP-HR$^{eba\text{-}175}$ transfections were PCR positive for integration (**Supplementary Fig. 3e**) at ~5 weeks post transfection. We selected one pUC19-sgRNA-T and two *eba-175*–sgRNA-T samples for Southern blot analysis. Consistent with the PCR test for integration, only the *eba-175*–sgRNA-T samples showed the expected insertion. Furthermore, in agreement with our previous experiment, one sample showed ~50% and the other ~80% editing at the *eba-175* locus (**Supplementary Fig. 3f**). Altogether, these data indicate that editing of endogenous loci in *P. falciparum* using the CRISPR-Cas9 system is highly efficient.

The potential for unintended gene disruptions due to induced off-target strand breaks and repair by the error-prone nonhomologous end joining (NHEJ) mechanism has been described in human cells[16–18]. We sought preliminary insight into how frequent such off-target events could be in *P. falciparum*, which lacks canonical NHEJ components[19]. A recent study examining chromosomal double-strand breaks induced by the meganuclease I-Sce1 showed that these are very efficiently and exclusively repaired by homologous donor sequence when present. In the absence of a suitable donor, however, an NHEJ-like repair process of unknown mechanism that resulted in elimination of the I-Sce1 target site was observed[20]. To understand how an off-target Cas9 and sgRNA-T–induced chromosomal double-strand break might be processed in *P. falciparum*, we simulated such an event by expressing the *kahrp*-sgRNA-T in the absence of a suitable homologous donor plasmid. Control parasites expressing pUC19-sgRNA-T were transfected in parallel. In two independent experiments, we observed no gross defects in relative growth between the two parasite lines generated, as both reached working parasitemias at similar times post transfection and qualitatively expanded comparably thereafter. To determine whether NHEJ-like events had occurred within the *kahrp* region targeted for cleavage, we deep-sequenced total genomic DNA from the *kahrp*-sgRNA-T– and pUC19-sgRNA-T–transfected lines (**Supplementary Fig. 4**). With more than 3×10^6 reads each from the *kahrp*- and pUC19-sgRNA-T experiments, we observed ~1,500 total indels in both test and control samples, which is indistinguishable from the expected background level of 2,000 indels at our read length and depth for the sequencing method. Presently, we do not understand why cleavage induced by Cas9-sgRNA versus I-Sce1 meganuclease in the absence of a suitable donor sequence produces different repair outcomes, but it is an active research area given the implications for improving genome-editing efficiency.

These findings promise to substantially accelerate the process for achieving targeted gene disruptions in *P. falciparum* for functional genetics studies.

METHODS

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

Accession codes. The sequences for plasmids reported in this study are deposited in GenBank/EMBL/DDBJ under KM099231–KM099240.

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

ACKNOWLEDGMENTS

We thank D. Taylor (University of Hawaii) for providing the anti-KAHRP mAb 89 antibody used for western blots and N. Watson (W.M. Keck Microscopy Facility at the Whitehead Institute) for technical assistance with SEM imaging. The following reagent was obtained through the MR4 as part of the BEI Resources Repository, NIAID, US National Institutes of Health (NIH): *P. falciparum* pfGNr malaria expression vector, MRA-462, deposited by C. Plowe. Quantitative PCR analyses were performed through the Genomics and Imaging Facilities Core of the MIT Center for Environmental and Health Sciences, which is supported by NIEHS Center Grant P30-ES002109. This work was supported by NIGMS Biotechnology Training Grant 5-T32-GM08334 (J.C.W.) and NIEHS Training Grant in Toxicology 5-T32-ES007020 (S.J.G.). This material is based upon work supported by the US National Science Foundation Graduate Research Fellowship under grant no. 1122374 (R.J.P.) and an NIH Director's New Innovator Award (1DP2OD007124) to J.C.N. and is funded by a grant from the Bill and Melinda Gates Foundation through the Grand Challenges Explorations initiative (OPP1069759) to J.C.N.

AUTHOR CONTRIBUTIONS

J.C.W. generated all plasmid reagents and transgenic parasites, and performed all experimental analyses of these lines. R.J.P. performed *in vitro* Cas9 cleavage assays and deep sequencing. S.J.G. collected SEM imaging data. J.C.W. and J.C.N. designed experiments and analyzed data. J.C.W. and J.C.N. wrote the manuscript with input from R.J.P. and F.Z. J.C.N. supervised the research.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Molecular biology. All plasmids used in this study were assembled using previously described methods[14]. Restriction enzymes and Gibson Assembly master mix were purchased from New England BioLabs. Primers used in this study are summarized in **Supplementary Table 1**.

Parasite culture and transfection. *P. falciparum* strain 3D7 parasites were grown under 5% O_2 and 5% CO_2 in RPMI-1640 medium supplemented with 5 g/L Albumax II (Life Technologies), 2 g/L sodium bicarbonate, 25 mM HEPES, pH 7.4 (pH adjusted with potassium hydroxide), 1 mM hypoxanthine and 50 mg/L gentamicin. For strains containing plasmids, appropriate selection drug combinations based on the markers used were added to medium as follows: 2.5 mg/L blasticidin-S and/or 250 mg/L G418 (Research Products International). Single and double vector transfections were carried out by the spontaneous uptake method[21] using ~50 µg of maxi-prepped plasmid DNA and eight square-wave electroporation pulses of 365 V for 1 ms each, separated by 0.1 s. Drug selection was initiated 4 d post transfection.

Assessing T7 RNAP expression in *P. falciparum*. 3D7 parasites were transfected with either pT7 RNAP (expresses T7 RNAP) or pfGNr (no T7 RNAP expression) plasmids and episomal lines selected using G418. In pT7 RNAP, T7 RNAP is expressed using the *PcDT* 5′ and *Pfhrp*2 3′ UTR pair, and selection with G418 is facilitated by *gfp-nptII* expressed from the *PfCam* 5′ and *Pfhsp86* 3′ UTR pair. To establish production of T7 RNAP protein, we saponin-lysed schizont-stage parasite–infected red blood cells transfected with either pT7 RNAP or pfGNr, and the lysate components were separated by SDS-PAGE. Western blot analysis was carried out using a mouse anti–T7 RNAP monoclonal primary antibody (Novagen; catalog #70566-3) at a 1:5,000 dilution in Tris-buffered saline, 0.1% Tween 20 and 5% milk, and an HRP-conjugated goat anti-mouse secondary antibody (Novagen, catalog #71045-3) at a 1:5,000 dilution. Blots were developed using the SuperSignal West Femto kit (Pierce).

Assessing T7 RNAP activity in *P. falciparum*. To assess T7 RNAP enzymatic activity, we cotransfected 3D7 parasites with pairs of either pT7 RNAP or pfGNr and pT7 RL1, pT7 RL2 and pΔT7 as indicated in the text. The pT7 RL1, pT7 RL2 and pΔT7 plasmids encode (i) a blasticidin-S deaminase (*bsd*) selection marker controlled by the *PfCam* 5′ and *Pfhsp86* 3′ UTR pair and (ii) a firefly luciferase gene controlled by the *PcDT* 5′ and *Pfhrp2* 3′ UTR pair. The latter is used to monitor the progress of transfections. After episomal lines were selected, total RNA for quantitative RT-PCR and northern blot analysis was isolated from saponin-lysed schizont-stage parasites. Pellets were stored in liquid nitrogen pending RNA extraction. RNA was extracted using Trizol (Life Technologies) and further purified using RNeasy purification kits (Qiagen).

For quantitative RT-PCR, extracted RNA was treated with Turbo DNA-free kit (Ambion) and reverse transcribed to cDNA using the SuperScript III First Strand Synthesis System (Invitrogen). cDNA was quantified using gene-specific primers (**Supplementary Table 1**) and SYBR Green on a LightCycler 480 Instrument II (Roche Applied Science). The thermocycling program used was as follows: initial denaturation at 95 °C for 5 min, followed by 45 cycles of 95 °C for 30 s, 60 °C for 30 s and 72 °C for 30s, followed by a melting-curve analysis. cDNA levels were quantified relative to standard curves generated using authentic plasmid templates.

Northern blot analysis was performed using the TurboBlotter Transfer System (Whatman), and the North2South Chemiluminescent Detection kit (Thermo Scientific) for development. Authentic RNA standards were synthesized by *in vitro* transcription using the MEGAscript T7 Transcription kit (Ambion).

Quantitative growth assays. Relative growth rates for the following dual-transfected parasite lines were determined: (i) pfGNr (No T7 RNAP) and pΔT7 (No T7 promoter–driven cassette), (ii) pfGNr and pT7 RL1, (iii) pT7 RNAP and pΔT7, and (iv) pT7 RNAP and pT7 RL1. Synchronized ring-stage parasites were set up in triplicate in 96-well microtiter plates and seeded at <2% parasitemia and 2% hematocrit in 200 µl of medium containing both G418 and blasticidin S. The pΔT7 plasmid, which confers blasticidin S resistance, was used in the no-pT7 RL1 cotransfections, whereas the pfGNr plasmid, which confers G418 resistance but does not express T7 RNAP, was used in the no-T7 RNAP expression conditions. This permitted coselection of all test lines with G418 and blasticidin S to eliminate this as a variable in the outcomes of the growth assays. Expansion was measured over four generations at the trophozoite stage. At each measurement, cultures were split to achieve an initial parasitemia of <2% to avoid overexpansion. Parasitemias were measured by fixing for ≥3 h in 1% formaldehyde acid citrate dextrose (ACD) buffer before SYBR Green I staining and analysis by flow cytometry (Accuri C6). Expansion rates were normalized to those of the control line (pfGNr and pΔT7, identified as '– pT7 RL1, – pT7 RNAP' in **Fig. 1e**) for each time point measured.

***In vitro* Cas9 cleavage assays.** *In vitro* cleavage assays were performed as previously described[22]. Briefly, HEK 293FT cells were transfected with the Cas9 expression plasmid pX165 (ref. 7) in six-well plates using Lipofectamine 2000 (Life Technologies) according to the manufacturer's protocol. Forty-eight hours post transfection, cells were lysed with 250 µl lysis buffer (20 mM HEPES, pH 7.5, 100 mM potassium chloride, 5 mM magnesium chloride, 1 mM dithiothreitol, 5% glycerol, 0.1% Triton X-100) supplemented with a protease inhibitor cocktail (Roche). Lysates were sonicated for 10 min and cell debris pelleted by centrifugation for 20 min at 5,000*g*. Lysates containing Cas9 protein were divided into aliquots and stored at −80 °C.

sgRNA-Ts were *in vitro* transcribed using the MEGAscript T7 kit (Ambion) using a circular plasmid template containing a cassette for T7 RNAP–dependent expression of the specific sgRNA as a template. The target DNA was amplified from *P. falciparum* genomic DNA with primers specific to the gene being tested (**Supplementary Table 1**). Cell lines have not been tested for *Mycoplasma* contamination.

CRISPR-Cas9 genome editing in *P. falciparum*. The pT7 RNAP-HR^{kahrp} and pT7 RNAP-HR$^{eba\text{-}175}$ plasmids were assembled as follows. The left homologous (LH) regions for *kahrp* and *eba-175* were amplified using the *kahrp*_LH_Forw/*kahrp*_LH_Rev and *eba-175*_LH_Forw/*eba-175*_LH_Rev primer pairs, respectively.

The corresponding right homologous (RH) regions were amplified using the *kahrp*_RH_Forw/*kahrp*_RH_Rev and *eba-175*_RH_Forw/*eba-175*_RH_Rev primer pairs. *kahrp*_T2a-*RL* and *eba-175*_T2a-*RL* coding regions were amplified using the *kahrp*_T2a-*RL*_Forw/*kahrp*_T2a-*RL*_Rev and *eba-175*_T2a-*RL*_Forw/*eba-175*_T2a-*RL*_Rev primer pairs, respectively. All PCR products were purified by agarose gel electrophoresis and extracted using the Qiaex II gel extraction kit (Qiagen). Equimolar mixtures of the *kahrp*_LH, *kahrp*_T2a-*RL* and *kahrp*_RH products and *eba-175* LH, *eba-175*_T2a-*RL* and *eba-175*_RH products were separately combined with Gibson Master Mix (New England BioLabs). After a 1-h incubation at 50 °C, a fraction of each crude reaction mixture was PCR amplified using the appropriate pair of *kahrp*_LH_Forw/*kahrp*_RH_Rev and *eba-175*_LH_Forw/*eba-175*_RH_Rev primers to produce the full length *kahrp*_LH-T2a-*RL*_*kahrp*_RH and *eba-175*_LH-T2a-*RL*_*eba-175*_RH repair cassettes. These products were purified by agarose gel electrophoresis and Qiaex II gel extraction, each combined with SalI-digested pT7 RNAP (~1:3 vector:product molar ratio) and assembled in a Gibson reaction. pCas9-*kahrp* sgRNA-T was constructed through a Klenow reaction on the annealed *kahrp*_gRNA_Forw and *kahrp*_gRNA_Rev primers followed by PCR amplification of this product using the *T7* promoter adaptor and *T7* terminator adaptor primers. This produced the required *T7* promoter–driven *kahrp* sgRNA-T expression cassette, which was inserted at the SalI site in the pCas9 vector through a Gibson reaction. To make pCas9–*eba-175* sgRNA-T, we used the *eba-175* sgRNA_Forw and *T7* terminator adaptor primers to PCR-amplify an *eba-175* sgRNA-T7 terminator fragment using the *kahrp* sgRNA-T expression cassette as a template. This product was PCR amplified using the *T7* promoter adaptor and *T7* terminator adaptor primers to produce a complete *T7* promoter–driven *eba-175*–sgRNA-T expression cassette, which was cloned into the SalI site of pCas9. All PCR amplification reactions were performed using a 15:1 (v:v) mixture of Hemo KlenTaq polymerase and Vent polymerase (New England BioLabs) in Hemo KlenTaq Buffer. Elongation was carried out at 60 °C for 2 min/kb of DNA to be amplified. To recover final plasmids, we transformed electrocompetent DH5α *Escherichia coli* cells with a portion of the Gibson reaction mixture and plated them on kanamycin-selective solid medium.

Plasmids encoding either T7 RNAP with homologous donor regions (pT7 RNAP-HR) or not (pT7 RNAP) and Cas9 with a sgRNA (pCas9-sgRNA-T) or not (pCas9-No sgRNA-T) were cotransfected as indicated into 3D7 and 3D7^{attB}::FLuc[14] (*kahrp* locus–targeted) or NF54^{attB} (ref. 23; *eba-175* locus–targeted) backgrounds. The NF54-attB line was obtained from D. Fidock (Columbia University). Cas9 and bsd selection marker expression are controlled by the *PcDT* 5′/*Pfhrp2* 3′ and *PfCam* 5′/*Pfhsp86* 3′ UTR pairs, respectively. In negative-control experiments, either no sgRNA or one targeting a region in the ampicillin resistance gene on the pUC19 plasmid and that is not present in the *P. falciparum* genome was used. Approximately 50 μg of each plasmid were cotransfected and simultaneously selected beginning at 4 d post transfection. Luciferase levels were measured periodically to determine whether genomic editing had occurred during experiments when the *kahrp* gene was targeted. Parasites were detectable by Giemsa smear within a typical 4- to 6-week transfection period. The analyses reported here were done over the ensuing several weeks. Main cultures were kept under drug selection, whereas no drug pressure was applied during clone isolation.

Analysis of CRISPR-edited parasite lines. Firefly and *Renilla* luciferase levels were measured using the Dual-Luciferase Reporter Assay System (Promega). Infected red blood cells were lysed using passive lysis buffer supplied with the kit, and measurements were made according to the manufacturer's instructions on a GloMax 20/20 luminometer (Turner Biosystems).

PCR analyses to assess modification of the targeted loci were carried out using a 15:1 (v:v) mixture of Hemo KlenTaq:Pfu Turbo (Agilent) in Hemo KlenTaq Buffer with genomic DNA purified from saponin-lysed cultures using the QIAamp DNA blood mini kit (Qiagen). The primers used are included in **Supplementary Table 1**.

Samples for KAHRP western blot analysis were obtained by repeated hypotonic lysis of infected red blood cells in water followed by high-speed centrifugation (21,000*g* for 1 min). The membrane fraction was recovered, solubilized in 1× SDS loading buffer and separated by SDS-PAGE. For KAHRP detection, a primary mouse monoclonal anti-KAHRP antibody[24] (mAb 89) provided by D. Taylor (University of Hawaii) was used at a 1:1,000 dilution in combination with an HRP-conjugated goat anti-mouse secondary antibody (Novagen; catalog #71045-3) at a 1:5,000 dilution. Blots were developed using the SuperSignal West Femto kit (Pierce). For NPTII detection, blots were probed with anti-NPTII antibody (Millipore 06-747) at a 1:1,000 dilution and developed with secondary antibody as described above.

Southern blots were carried out on DNA purified from infected red blood cells using the QIAamp DNA blood mini kit (Qiagen) after saponin lysis. Samples were restriction enzyme–digested overnight with HindIII, BamHI and PstI, and HindIII and XbaI for analysis of the *kahrp* and *eba-175* loci, respectively. Blots were processed using the TurboBlotter kit (Whatman) for transfer and the North2South kit (Thermo Scientific) for development.

Scanning electron microscopy (SEM) was performed as described[25] on a JEOL 5600LV SEM instrument operated at 10 kV.

Deep sequencing. We performed deep sequencing as previously described[26]. Briefly, the target site within the *kahrp* gene was amplified by PCR to include generic handles. Using the handles as priming sequences, we performed a second round of PCR to include unique barcodes for sample identification as well as Illumina P5 and P7 adaptors. PCR products were pooled in an equimolar ratio and purified by the QIAquick Gel Extraction Kit (Qiagen). Barcoded and purified pooled sequencing libraries were quantified using the Qubit dsDNA HS Assay Kit (Life Technologies) and sequenced using the Illumina MiSeq Personal Sequencer (Life Technologies).

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doi:10.1038/nmeth.3063