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A Cas9 transgenic *Plasmodium yoelii* parasite for efficient gene editing

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ABSTRACT

The RNA-guided endonuclease Cas9 has applied as an efficient gene-editing method in malaria parasite *Plasmodium*. However, the size (4.2 kb) of the commonly used Cas9 from *Streptococcus pyogenes* (SpCas9) limits its utility for genome editing in the parasites only introduced with cas9 plasmid. To establish the endogenous and constitutive expression of Cas9 protein in the rodent malaria parasite *P. yoelii*, we replaced the coding region of an endogenous gene *sera1* with the intact SpCas9 coding sequence using the CRISPR/Cas9-mediated genome editing method, generating the cas9-knockin parasite (*PyCas9ki*) of the rodent malaria parasite *P. yoelii*. The resulted *PyCas9ki* parasite displays normal progression during the whole life cycle and possesses the Cas9 protein expression in asexual blood stage. By introducing the plasmid (pYCs) containing only sgRNA and homologous template elements, we successfully achieved both deletion and tagging modifications for different endogenous genes in the genome of *PyCas9ki* parasite. This cas9-knockin *PyCas9ki* parasite provides a new platform facilitating gene functions study in the rodent malaria parasite *P. yoelii*.

1. Introduction

Malaria remains the one of the most deadly infectious diseases worldwide. To uncover molecular targets for the development of anti-malaria medicine and for studying disease mechanism, new technologies and methods are urgently needed [1,2]. CRISPR/Cas9 (clustered regularly interspaced short palindromic repeats /CRISPR-associated protein 9), a powerful genome editing technology, has been successfully adapted for genome modification of malaria parasites, including *Plasmodium falciparum* and *Plasmodium yoelii* [3–6]. The CRISPR/Cas9 system was originated from a prokaryotic RNA programmable nuclease that can introduce a double-strand break (DSB) at a specific site on a chromosome through heterologous expression of two components: Cas9 endonuclease and a targeting single guide RNA (sgRNA) [7]. The DNA DSB is repaired primarily by homologous recombination (HR) pathway in *Plasmodium* [8,9], therefore, the donor templates are needed for the CRISPR/Cas9-mediated genome editing practices. For editing the genome of *Plasmodium falciparum*, the Cas9/sgRNA cassette and donor templates are delivered into parasites in two separate vectors with each plasmid carrying a unique drug resistant gene as selection marker [3,5,10–12]. For the rodent malaria parasite *Plasmodium yoelii*, a single vector system is used because limited independent drug selection markers are available for the parasites [4,13]. In the one-vector plasmid (pYC) design, all components, including the genes encoding the Cas9 protein, the human dihydrofolate reductase (hDHFR) for positive selection with pyrimethamine (Pyr), the sgRNA, and the donor template DNAs are included. The *Streptococcus pyogenes*-derived Cas9 (SpCas9) has been used for CRISPR/Cas9-mediated gene editing in all malaria parasites [3–5]; however, the *SpCas9* is approximately 4200 bp in size, which significantly increases the plasmid size and the difficulty in cloning all the required elements in a single plasmid [7]. Recently, a constitutive Cas9 expression was established to maximize the CRISPR/Cas9-mediated gene disruption and achieve genome-wide gene screening in another apicomplexan *Toxoplasma gondii* [14].

To improve gene editing efficiency and to facilitate the plasmid construction, we generated a Cas9-knockin *P. yoelii* parasite that can constitutively express Cas9 endonuclease. We replaced the gene encoding *P. yoelii* serine repeat antigen 1 (SERA1) with an intact SpCas9 coding sequence using the CRISPR/Cas9-mediated genome editing method, generating a cas9-knockin parasite (*PyCas9ki*). The resulting *PyCas9ki* parasite displays normal progression during the whole life cycle and expresses the Cas9 protein in asexual blood stages. By introducing the plasmid (pYCs) containing only sgRNA and homologous template elements, we successfully achieved both deletion and tagging modifications for several genes in the genome of *PyCas9ki* parasite. This

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cas9-knockin *PyCas9ki* parasite provides a new platform facilitating studies of gene function in *P. yoelii*.

2. Materials and methods

2.1. Plasmid construction

The procedures to construct plasmid vectors for gene deletion, tagging, and replacement were as described previously [4]. Briefly, to construct pYCm vector for deleting the coding region of *sera1* gene (PY17X_0305400), we amplified 526 bp 5′ untranslated region (UTR) upstream of translation start codon as the left homologous arm and a 500 bp 3′UTR region following translation stop codon as right homologous arms using PCR primers listed in Table S1. One sgRNA was designed to target a site in the coding region to be deleted. All the fragments were sequentially ligated into the pYCm vector using T4 ligase. To clone the *SpCas9* coding sequence into the pYCm vector for replacing the coding region of *sera1* gene, we amplified the full-length *SpCas9* coding region with nuclear localization signal at both 5′ and 3′ flank from the plasmid pYC [4], tagged the sequence with a quadruple Myc epitope (4Myc) at C-terminal, and inserted it into the pYCm vector between the left and right homologous arms. For gene editing purpose in the *PyCas9ki* parasite, we engineered an plasmid pYCs derived from the original pYCm, where the Cas9 encoding region was removed *via* mutagenesis PCR, and the sgRNA cassettes, the multiple cloning site, and the *hdhfr/yfcu* selection marker were sustained. The resulting pYCs vector was then used to make constructs for deleting the *cdpk3* gene (PY17X_0410700) and *ctrp* gene (PY17X_0415800), and for tagging the *sep1* (PY17X_0526200) and *dhhc10* genes (PY17X_0946500), respectively, using similar procedures. All the primers and oligonucleotides used are listed in Table S1.

2.2. Malaria parasite and parasite transfection

The *P. yoelii* 17XNL strain was propagated in ICR mice (female, 5–6 weeks old) purchased from the Animal Care Center, Xiamen University. All mouse experiments were performed in accordance with approved protocols (XMULAC20140004) by the Committee for the Care and Use of Laboratory Animals at the School of Life Sciences, Xiamen University. All transfections were performed on the 17XNL parasite or *PyCas9ki* strain. The procedures for parasite transfection, Pyr selection, and cloning were as described previously [4]. Briefly, parasites were electroporated with purified plasmid DNA using Amaxa Nucleofector™ (Lonza, Switzerland). Transfected parasites were immediately intravenously injected into a naive mouse. Pyr (6 ug/ml) supplied in drinking water was provided to mice for drug selection from day 2 post-transfection. A small amount of blood sample was taken daily through tail clip and Giemsa-stained for infected red blood cells (iRBCs). Pyr resistant parasites usually appear 5–6 days after drug selection.

2.3. Negative selection with 5-Fluorocytosine

To remove the plasmid inside the parasite from the first round transfection, parasites were subjected to negative selection with 5-Fluorocytosine (5-FC) as described previously [13]. Briefly, 5-FC (Sigma, USA) was prepared in water at a final concentration of 2.0 mg/ml and was provided to the animals in a dark drinking bottle. A naïve mouse receiving parasites containing residual plasmids after Pyr selection was subjected to 5-FC pressure for 8 days, with a change of new drug at day 4. Complete removal of plasmids in parasites was confirmed by PCR genotyping.

2.4. DNA preparation and detection of genetic modifications

Blood samples from infected mice were collected from the orbital sinus, and RBCs were lysed with 1% saponin in PBS. Parasite genomic DNAs were isolated using DNeasy Blood kits (Qiagen) after washing off hemoglobin and were used in PCR amplifications. For gene deletion and gene tagging, targeted modification was confirmed by PCR using two pairs of primer to detect 5′ and 3′ integrations respectively. To confirm the successful deletion of targeting region, another independent primer pairs were designed to amplify the region to be deleted. All the primers used are listed in Table S1.

2.5. Gametocyte induction and in vitro ookinete differentiation

Ookinetes were prepared according to the procedure described previously [15]. Briefly, infected blood was injected intraperitoneously into mice that were made anemic by phenylhydrazine treatment (80 μg drug per mouse body weight) over a three-day period. Three days after infection, 200 μl of infected blood containing gametocytes was obtained from the orbital sinus and mixed immediately with 1 ml ookinete culture medium. The mixture was incubated at 22 °C for 20–24 h to allow gametogenesis, fertilization, and ookinete differentiation. Ookinetes formation was monitored by Giemsa staining of smears of the cultured cells.

2.6. In vitro ookinete gliding motility assay

Ookinete gliding motility was evaluated as previously described [15,16]. All procedures were performed in a temperature (22 °C)-controlled room. Twenty μl of the ookinete cultures was mixed with an equal volume of Matrigel (BD, USA) on ice. The mixture was transferred on a slide, sealed with nail varnish after adding a coverslip, and left at 22 °C for 30 min before microscopy analysis. Time-lapse videos (1 frame every 20 s, for 20 min) were taken to monitor ookinete movement using a 40 × objective lens on a Nikon ECLIPSE E100 microscope fitted with an ISH500 digital camera controlled by the ISCapture v3.6.9.3_N software (Tucsen, CHINA). Time-lapse movies were analyzed with Fiji and the Manual Tracking plugin. Ookinete gliding speed was calculated by dividing the distance that each individual ookinete moved by the tracking time. The experiments were performed in three times independently.

2.7. Mosquito infection and observation of parasites in mosquitoes

For mosquito infection, 30–40 female *Anopheles stephensi* mosquitoes were allowed to feed on one anaesthetized infected mice that carried comparable numbers of gametocytes as determined by Giemsa staining for 20 min. 15–20 mosquitos were dissected day 7 post-infection, and oocysts in the midguts were counted. Salivary glands were isolated from 15 to 20 dissected mosquitos 14 days post-infection, and sporozoites were collected and counted using a hemocytometer under the microscopy.

2.8. Sporozoite infectivity assay

One ICR mouse was anesthetized and exposed to 10–15 infected mosquitoes. Mosquitoes were allowed to bite the mouse for 20 min. The time to detect the blood stages parasite in the mouse after mosquito bite (prepatent time, defined as > 1 infected erythrocyte/10,000 erythrocytes) was determined microscopically using Giemsa-stained thin blood smears. All mice were monitored daily for 14 days post mosquito bite.

2.9. Immunofluorescence assay

Parasite samples are harvested by centrifuging for 3 min at 2000 rpm, washed twice with PBS, and suspended in 4% freshly prepared paraformaldehyde on a poly-L-lysine coated glass slide for 15 min. Cells were washed twice with PBS, and permeabilized with 0.1% Triton X-100 for 8 min at room temperature. The sample was

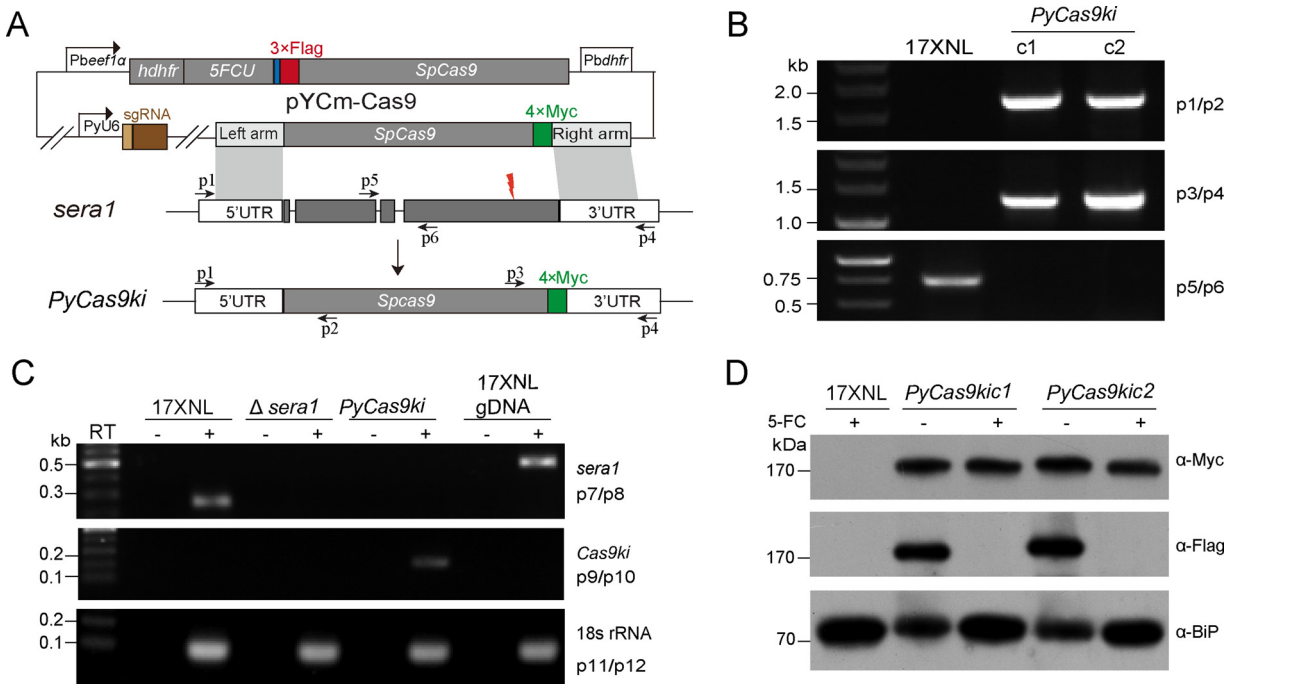

Fig. 1. Generation of a *Plasmodium yoelii* parasite expressing Cas9 protein.

A. Diagram of CRISPR/cas9 plasmid pYCm construct for replacing the endogenous *sera1* coding region with the *cas9* gene. The red thunderbolt indicates sgRNA recognition site. The *Cas9* sequence tagged with quadruple Myc epitope (4Myc, green) is to be integrated into parasite genome, whereas the *Cas9* sequence tagged with triple Flag epitope (3Flag, Red) functions to generate targeted double strand break for CRISPR/cas9 mediated modification.

B. Diagnostic PCR of two transgenic parasite clones (*PyCas9kic1* and *PyCas9kic1*) showing expected modification in *sera1* locus. Primers (p) used are shown in (A) and listed in Table S1.

C. RT-PCR detection of both *sera1* and *cas9* mRNAs expression in the asexual blood stage of 17XNL, Δ*sera1*, and *PyCas9ki* parasites. PCR amplifications of the cDNA from the total RNA with (+) or without (−) reverse transcription are indicated. *18s rRNA* mRNA serves as the internal control.

D. Western blotting analysis of Cas9 protein expression in wildtype 17XNL and *PyCas9ki* parasite clones. Anti-Flag or anti-Myc antibodies were used to detect the expression of the 3Flag::Cas9 or Cas9::4Myc indicated in (A), respectively. BiP protein serves as an internal control. Parasite clones were treated with (+) or without (−) 5-FC to remove the pYCm plasmid episome (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

incubated with primary antibodies (rabbit anti-HA, Cell Signaling Technology, RRID:AB_1549585, 1:500; rabbit anti-Myc, Cell Signaling Technology, RRID:AB_10692100, 1:500; rabbit anti-Flag, Sigma–Aldrich, RRID:AB_796202, 1:500) in 5% BSA in PBS for 12 h at 4 °C, washed with PBS three times, and incubated with fluorescence conjugated secondary antibodies (Alexa 488 goat anti-mouse IgG secondary antibody, ThermoFisher Scientific, RRID:AB_2534069, 1:2000; Alexa 555 goat anti-rabbit IgG secondary antibody, ThermoFisher Scientific, RRID:AB_141784, 1:2000) RT for 1 h. Subsequently, the cells were washed three times with PBS, and stained with Hoechst33342 (ThermoFisher Scientific, 1:5000) for 8 min, mounted in 90% glycerol solution, and sealed with nail polish. All images were captured using identical settings in the Zeiss LSM 780 laser scanning confocal microscope with a 100× oil objective. Results were obtained from three independent experiments.

2.10. Western blotting

Protein extraction was performed using RIPA lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 2 mM sodium pyrophosphate, 25 mM β-glycerophosphate, 1 mM EDTA, 1 mM Na_3VO_4, 0.5 μg/ml leupeptin) plus 1 × complete protease inhibitor cocktail and 1 mM PMSF. The parasite cell extracts was incubated on ice for 30 min and centrifuged at 12,000*g* for 10 min at 4 °C. Supernatant was collected and mixed with 5× protein loading buffer. Approximately 40 μg total protein was loaded and separated in 4–9% SDS-PAGE, transferred to a 0.22 μm PVDF membrane and blocked with 5% skim milk for 1 h. The binding of proteins was detected using rabbit anti-HA antibody (1:1000), rabbit anti-Myc antibody (1:1000), or rabbit anti-Flag antibody (1:1000), followed by a horseradish peroxidase (HRP)-conjugated rabbit secondary antibody (Abcam, RRID:AB_955447, 1:5000) and HRP-conjugated mouse secondary antibody (Abcam, RRID:AB_955439, 1:5000). A control lane on the blot was overlaid with rabbit antiserum against BiP (1:1000) produced in the lab. The proteins were detected using an enhanced chemiluminescent substrate kit (ECL) and visualized by the film at dark room.

2.11. RNA preparation and RT-PCR

Parasite pellets after lysis of RBCs were mixed with TRIzol (Invitrogen), and RNA was extracted according to the manufacturer's instructions. Purified RNA was treated with DNase using Turbo DNA-free kit (Life Technologies). cDNA was synthesized from 1 μg RNA according to the manufacturer's instructions (ThermoFisher Scientific). Unique primers were designed for reverse transcription or for amplifying short regions of target genes. The primers used in RT-PCR analysis are listed in Table S1.

2.12. Software and statistical analysis

Cas9/sgRNA targeting sites were designed using Eukaryotic Pathogen CRISPR guide RNA/DNA Design Tool (http://grna.ctegd.uga.edu). Image J (https://imagej.nih.gov/ij) was used to quantify fluorescent signals from imaging and to process images of ookinete gliding movement. GraphPad Prism5 (https://www.graphpad.com) was used

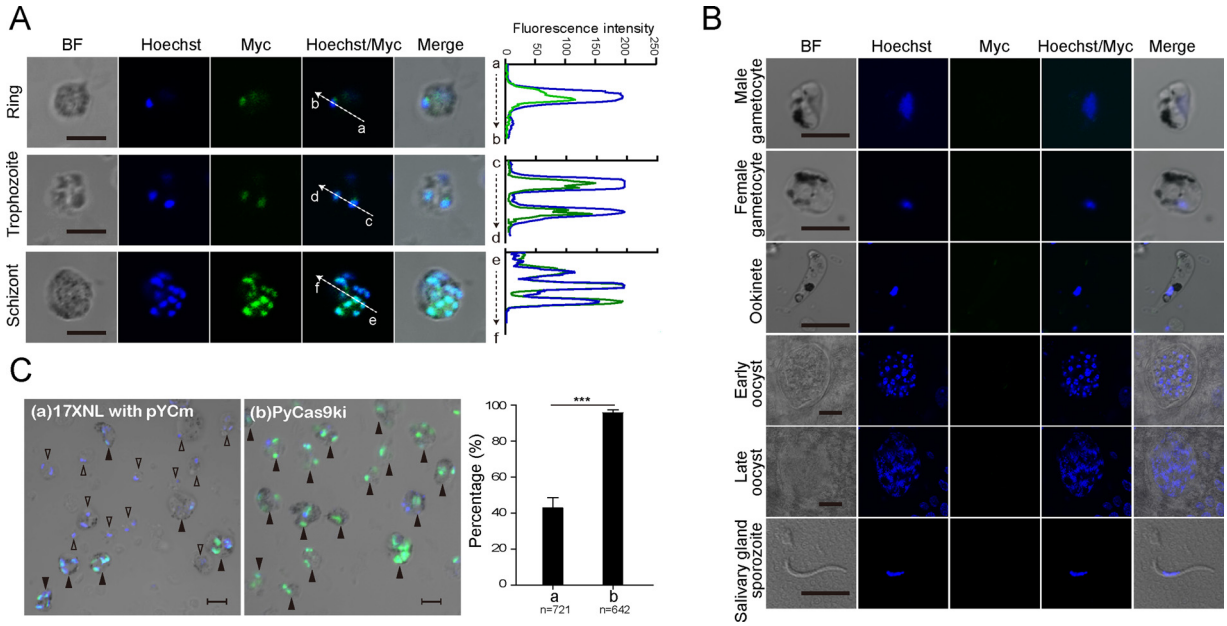

Fig. 2. Expression and localization of Cas9 protein in the *PyCas9ki* parasite.

A. Immunofluorescence assay (IFA) of Cas9 protein expression in asexual blood stages of the *PyCas9ki* parasite, including ring, trophozoite, and schizont. Nuclei were stained with Hoechst33342 (blue). Bar = 5 μm. Results are representative of three independent experiments. Overlapping peaks of co-localization between Cas9 protein and nucleus are shown in the right panel.

B. IFA analysis of Cas9 protein expression in sexual and mosquito stages of the *PyCas9ki* parasite, including male and female gametocytes, ookinetes, day 7 and day 14 oocysts, and salivary gland sporozoites. Bar = 5 μm.

C. IFA analysis of Cas9 protein expression in the asexual blood stage of 17XNL parasite transfected with pYCm plasmid and the *PyCas9ki* parasite. Parasite nucleuses are stained with Hoechst33342 (blue). Parasites with or without Cas9 expressing are indicated with ▲ or △, respectively. Bar = 5 μm. Quantification results were shown in the right panel. n is the number of cells counted in each group (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

for statistical analysis. Two-tailed Student's *t*-test or Whiney Mann test was used to compare differences between treated groups and their paired controls.

3. Results

3.1. Generation of Cas9 knockin transgenic parasite

Because the *Spcas9* gene is a 4200 bp coding sequence in size, it is challenging to incorporate the Cas9 coding sequence, its promoter and polyadenlyation sequence into the parasite genome together. We decided to replace the coding region of a non-essential gene, *sera1* [4,17], with *Spcas9* gene. To confirm that *sera1* is not essential for the parasite, we first disrupted the *sera1* gene in the 17XNL parasite using CRISPR/Cas9 methods described previously [13], and obtained two parasite knockout clones (Δ*sera1c1* and Δ*sera1c2*) with deletion of the whole coding region (Fig. S1A, B). Both Δ*sera1c1* and Δ*sera1c2* parasite clones displayed normal progression comparable with wildtype parasite in the life cycle, including asexual and sexual gametocytes stages in mice, mosquito stages, and mouse infectivity (Fig. S1C to G), suggesting functional redundancy of the *sera1* gene in the life cycle of *P. yoelii*.

Next, we constructed a plasmid pYCm-Cas9 containing an intact Cas9 encoding sequence tagged with quadruple Myc epitope (4Myc) C-terminally and flanked by two homologous regions of *sera1* (0.5 kb of the 5′-flanking region and 0.5 kb of the 3′-flanking region) (Fig. 1A). After transfection, drug selection, and parasite cloning, we obtained two parasite clones (*PyCas9kic1* and *PyCas9kic2*) with targeted integration of the *Spcas9* gene into the *sera1* locus (Fig. 1B). Correct replacement of *sera1* with *Spcas9* was further confirmed by RT-PCR analyses showing *cas9* transcript, but not that of *sera1* in the red blood stages of *PyCas9ki* parasites (Fig. 1C). Furthermore, expressions of both Cas9::4Myc protein from integrated gene and 3Flag::Cas9 protein from the episomal pYCm plasmid were detected on western blotting (Fig. 1D). To remove the episome plasmid in the parasites for next-round genetic modification, we applied the negative selection to the *PyCas9ki* parasite by using 5-FC [13] and confirmed the successful removal of episomal plasmid as no expression of 3Flag::Cas9 protein was detected in the parasites after 5-FC treatment (Fig. 1D).

To evaluate the effect of Cas9 expression on the parasite development and differentiation, we performed detailed analyses comparing the life cycle between wildtype and *PyCas9ki* parasites. *PyCas9ki* parasites displayed similar asexual proliferation and gametocytes formation in mouse blood stage (Fig. S2A, S2B), ookinete differentiation *in vitro* (Fig. S2C), day 7 oocysts per mosquito (Fig. S2D), day 14 salivary gland sporozoites (Fig. S2E), and sporozoite infectivity of mice to those of the WT parasite, suggesting that replacement of *sera1* with *Spcas9* does not affect parasite development.

3.2. Expression of Cas9 protein in asexual blood stage

The expression of 4Myc-tagged Cas9 protein was detected within the nucleus of different asexual blood stages of *PyCas9ki* parasites, including rings, trophozoites, and schizonts using anti-Myc antibody by immunofluorescence assay (IFA) (Fig. 2A). In addition, no expression of Cas9 protein was detected in gametocytes, ookinetes, midgut oocysts, and salivary gland sporozoites (Fig. 2B). These results indicated that the promoter of endogenous *sera1* gene possesses the transcription activity in the asexual blood stages of *P. yoelii* parasite. Interestingly, we observed significantly higher percentage of parasite cells expressing Cas9 protein in the asexual stage of *PyCas9ki* parasite over the parasite with episomal pYCm-Cas9 plasmids (Fig. 2C).

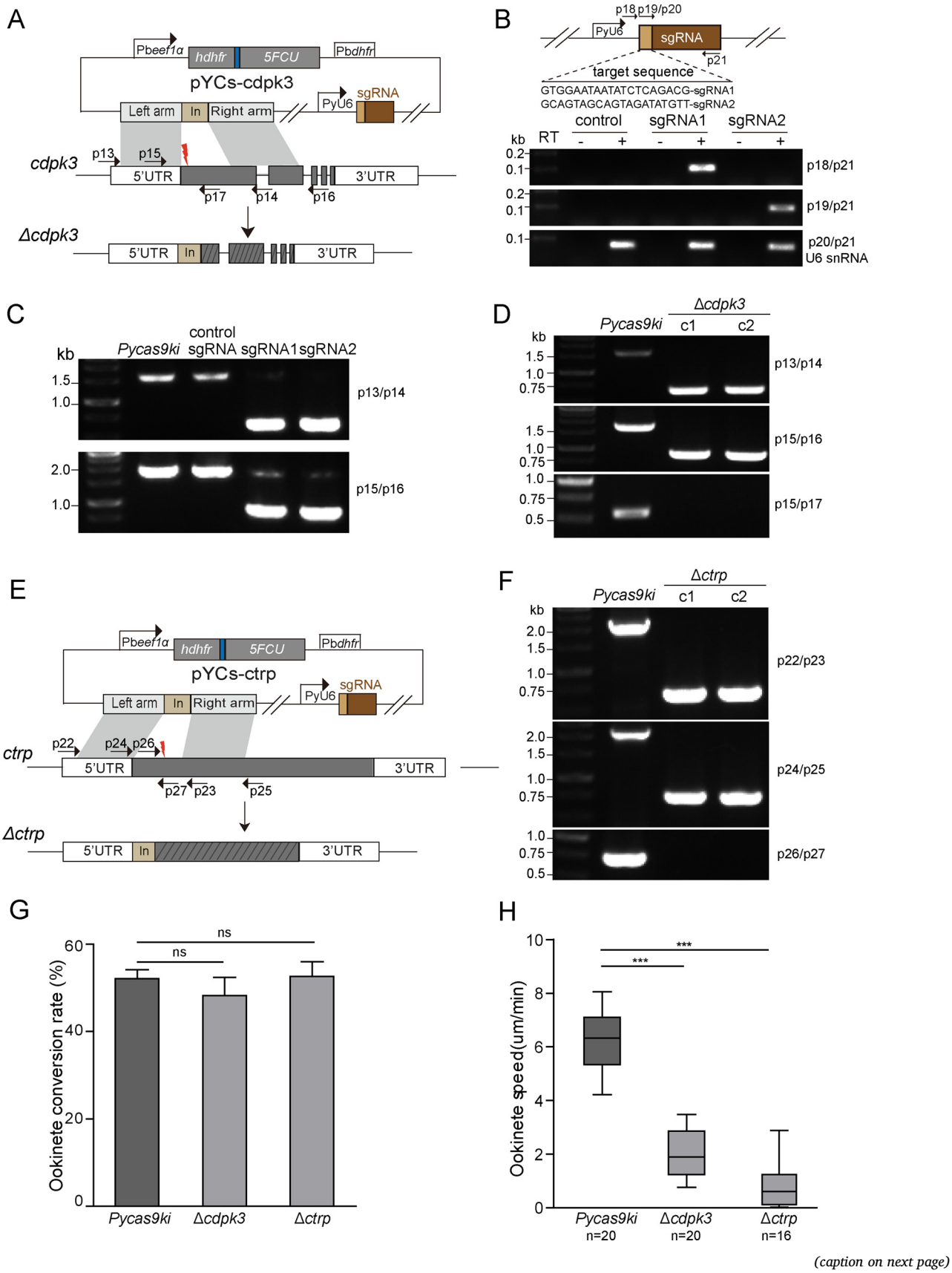

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Fig. 3. Deletion and mutant phenotype of endogenous *cdpk3* and *ctrp* genes in the *PyCas9ki* parasite.
A. Diagram of pYCs construct for deleting the endogenous *cdpk3* gene. The red thunderbolt indicates the sgRNA recognition site.
B. Schematic of sgRNA expressing cassettes driven by *P. yoelii* U6 snRNA promoter in the pYCs plasmid. The protospacer sequences of sgRNA1 and sgRNA2 are indicated. sgRNA transcripts are detected by RT-PCR using specific primer pair (p18/p21 and p19/p21) listed in Table S1. Endogenous U6 snRNA transcript serves as an internal control.
C. PCR analysis of genomic DNA from the 17XNL, *PyCas9ki*, and *PyCas9ki* parasites transfected with different sgRNA plasmids as indicted. Successful integration of left and right homologous arms was detected at specific sites in the parasite directed by both sgRNA1 and sgRNA2, but not control sgRNA targeting irrelevant sequences.
D. PCR analysis of two clones with *cdpk3* deletion in the *PyCas9ki* parasite.
E. Diagram of pYCs construct for deleting the endogenous *ctrp* gene.
F. PCR analysis of two clones with *ctrp* deletion in the *PyCas9ki* parasite.
G. *In vitro* ookinete differentiation of the parental *PyCas9ki* and mutant parasites.
H. Ookinete gliding motility of the parental *PyCas9ki* and mutant parasite using Matrigel motility assay. n is the number of ookinete tested in each group. Results are representative of two independent experiments (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

3.3. Endogenous gene deletion in the PyCas9ki parasite

Because the constitutive expression of Cas9 in the *PyCas9ki* parasite, only sgRNA cassette and homologous DNA templates are needed for editing a specific parasite locus. We next removed the *SpCas9* coding sequence in the pYCm vector *via* mutagenesis, resulting in a smaller plasmid, pYCs (Fig. S3). To test whether the endogenously expressed Cas9 protein could function in CRISPR/Cas9-mediated gene modification, we attempted to delete two genes (*ctrp* and *cdpk3*) in the genome of the *PyCas9ki* parasites, separately. The *ctrp* and *cdpk3* genes in both *Plasmodium berghei* and *P. yoelii* parasites were previously disrupted, leading to complete loss or severe defect in ookinete gliding motility and absence of oocysts in the mosquito after infection [13,18–21]. We constructed a plasmid pYCs-cdpk3 containing a 46-bp tag DNA (for PCR primers) flanked by two homologous regions of *cdpk3* (0.49 kb of the 5′-flanking region and 0.53 kb of the 3′-flanking region) (Fig. 3A). Two sgRNAs targeting the exon1 and exon 2 of the *cdpk3*, respectively, were designed and inserted into the pYCs-cdpk3 vector, generating plasmids pYCs-cdpk3-sgRNA1 and pYCs-cdpk3-sgRNA2. One day after electroporation of the plasmids into the *PyCas9ki* parasite, parasites were selected with Pyr supplied in drinking water. Pyr-resistant parasites were observed microscopically 5 to 6 days after electroporation. Expression of sgRNA1 and sgRNA2 transcripts was detected using RT-PCR in the transfected parasites (Fig. 3B). PCR analysis of genomic DNA from parental strain *PyCas9ki* and plasmid-transfected parasites indicated successful integration of left and right homologous arms at specific sites directed by both sgRNA1 and sgRNA2, but not by control sgRNA targeting irrelevant sequences (Fig. 3C). After limiting dilution cloning, two parasite clones with disrupted *cdpk3* gene were obtained and confirmed by PCR genotyping (Fig. 3D). Using the same method, we successfully disrupted the *ctrp* gene in the *PyCas9ki* parasites (Fig. 3E) and obtained two resulting mutant clones with targeted gene deletion (Fig. 3F). Both *PyCas9ki* parasite derived mutant clones (Δ*cdpk3* and Δ*ctrp*) displayed normal asexual growth and gametocyte formation in the mouse (data not shown), and comparable conversion rate to mature ookinetes (Fig. 3 G), but severe defect in ookinete gliding motility *in vitro* (Fig. 3F), which is consistent with both phenotypes of *cdpk3* and *ctrp* knockout in *P. yoelii* 17XNL parasite [13]. These results confirmed that successful gene deletion could be achieved *via* CRISPR/Cas9 in the *PyCas9ki* parasite in conjugation with pYCs plasmid system.

3.4. Tagging endogenous gene with epitope in the PyCas9ki parasite

Tagging endogenous genes with fluorescent proteins or epitope tags is widely used for studies of protein expression, localization and interaction. To test this application in the *PyCas9ki* parasite, we built a construct (pYCs-sep1::6HA) containing a 498 bp C-terminal region of the *sep1* gene followed by a sextuple HA tag (6HA) and a 512 bp 3′-flanking region (3′-UTR) of the *sep1* gene (PY17X_0526200) (Fig. 4A). The *sep1* gene encodes an early transcribed membrane protein locating at the parasitophorous vacuole membrane (PVM) [22]. We detected the integration of both donor templates into the 3′ end of *sep1* gene in the parasite 6 days after transfection and obtained two clones after limiting dilution cloning (Fig. 4B). The expression of recombinant Sep1::6HA protein was detected using western blotting in the asexual blood stage of both parasite clones (Fig. 4C). In asexual blood stages, the Sep1::6HA protein is localized at the PVM (Fig. 4D), which is consistent with the protein localization reported previously [22].

In addition, we attempted to knock in a 4Myc epitope tag in another gene *dhhc10* (PY17X_0946500) (Fig. 4E), the orthologue of which encodes a *S*-acyl-transferase that is expressed only in gametocytes and ookinetes of *P. berghei* [23]. Targeted knockin of the 4Myc tag at the 3′ end of *dhhc10* coding region was detected in the two parasite clones using genotypic PCR (Fig. 4F). Furthermore, the expression of recombinant DHHC10::4Myc protein was also detected in ookinetes of the two parasite clones on Western blotting (Fig. 4G). In ookinetes, the DHHC10::4Myc protein is expressed in distinct cytoplasmic foci, the ookinete specific organelle crystalloid body (Fig. 4H), consisting with previous observing [23]. Together, these results show that tagging endogenous genes can be achieved *via* CRISPR/Cas9 in the *PyCas9ki* parasite.

4. Discussions

In this study, we generate a transgenic rodent malaria parasite *P. yoelii* 17XNL *PyCas9ki* that expresses SpCas9 in asexual blood stages. The resulting *PyCas9ki* parasite displays normal progression during the whole life cycle. Furthermore, we demonstrate that both gene disruption and gene tagging, the most commonly used gene editing for gene function study, can be efficiently achieved in this *PyCas9ki* parasite without inclusion of *Spcas9* gene in the plasmid vector. This parasite will be useful for genetic modification of other genes in the genome without the need to include the *Spcas9* gene in the plasmid vector.

Several CRISPR/Cas9-based applications have been described for editing genomes of *Plasmodium* parasites [3–5,10–13]. In these Cas9 vector-based practices, the expression of exogenous Cas9 was driven by the promoters such as promoters from genes of *P. falciparum hsp86* and *P. berghei eef1αa*. These promoters possess the transcription activity for the whole parasite life cycle, including in sexual and mosquito stages. Currently, the asexual blood stages are the only stages that can be genetically manipulated efficiently [2]. An idea design for exogenous Cas9 expression would be to express the Cas9 protein in asexual blood stages only because of potential off-target effect by the endonuclease Cas9 [24,25]. In this study, we successfully generated a parasite that expresses the Cas9 protein in the asexual blood stages, including the ring, trophozoite, and schizont (Fig. 2A), but not in gametocyte, ookinete, oocyst, and sporozoite, preventing Cas9 derived off-target effects in these stages during the life cycle.

In our previous studies, we developed a CRISPR/Cas9-based single vector system pYC/pYCm to successfully modify *P. yoelii* genome,

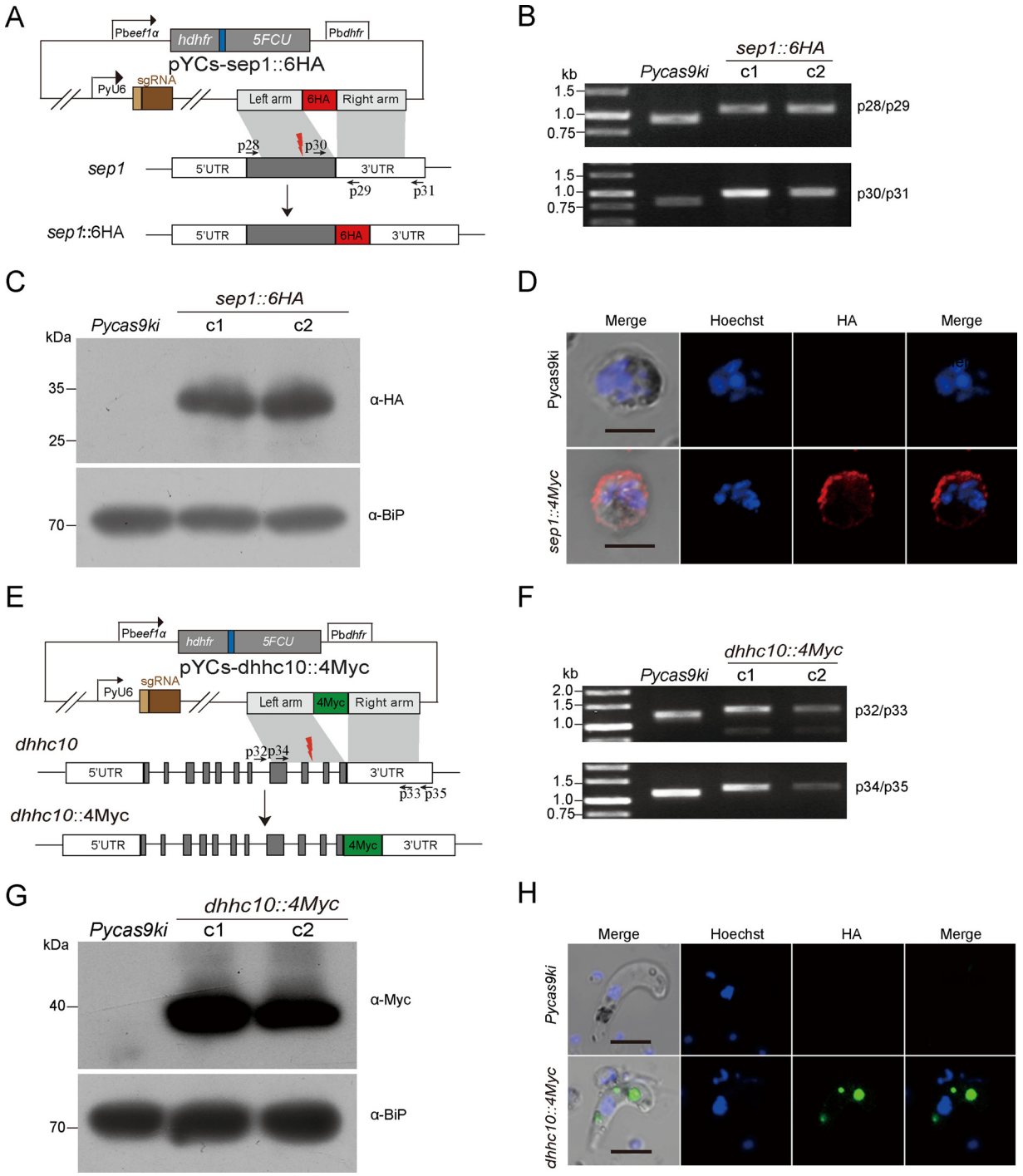

Fig. 4. Tagging and protein expression of endogenous *sep1* and *dhhc10* genes in the *PyCas9ki* parasite.
A. Diagram of pYCs construct for tagging the *sep1* gene with 6HA. The red thunderbolt indicates the sgRNA recognition site.
B. PCR detection of the *PyCas9ki* parasite and two *PyCas9ki* derived clones with the *sep1* gene tagged with 6HA at C-terminal.
C. Western blotting analysis of 6HA tagged SEP1 protein expression in the asexual blood stages of two *PyCas9ki* derived clones using anti-HA antibody. BiP protein serves as an internal control.
D. IFA analysis of 6HA tagged SEP1 protein expression in asexual blood stages of one *PyCas9ki* derived clone. Nuclei are stained with Hoechst33342. Bar = 5 μm.
E. Diagram of pYCs construct for tagging the *dhhc10* gene with 4Myc.
F. PCR detection of the *PyCas9ki* parasite and two *PyCas9ki* derived clones with *dhhc10* gene tagged with 4Myc at C-terminal.
G. Western blotting analysis of the 4Myc tagged DHHC10 protein expression in the ookinetes of two *PyCas9ki* derived clones using Anti-Myc antibody. BiP protein serves as an internal control.
H. IFA analysis of the 4Myc tagged DHHC10 protein expression in the ookinetes of one *PyCas9ki* derived clone. Nuclei were stained with Hoechst33342. Bar = 5 μm
(For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

including gene deletion, gene tagging, and nucleotide replacement [4,13]. In this one-vector plasmid pYCm design (Fig. S3), all components, including Cas9 and sgRNA expression cassettes, the multiple cloning site for insertion of donor template DNA, and drug selectable markers (*hdhfr* and *yfcu* for sequential positive and negative selection) were included. With all the elements included, the size of plasmid is large, making it difficult to maintain and propagate in bacteria. Compared with the size (10.0 kb) of pYCm plasmid, this new Cas9-free plasmid pYCs (5.7 kb) thus permit introduction of larger donor DNA sequences for more complicated genome editing.

In addition to gene deletion and gene tagging for the single endogenous gene, a particularly exciting category of future application will be the gene editing in multiple genes or loci simultaneously in the genome of this *PyCas9ki* parasite. The use of *PyCas9ki* parasite in conjunction with the pYCs vector containing multiplex sgRNAs and their responding donor templates for homologous recombination repair may make it feasible to modify more than one gene at a time. As multigene interactions play an important role in many biological processes of eukaryotic organisms, modification of multi-genes may be necessary in order to dissect the complex biological processes and gene functions in the development and differentiation of malaria parasite.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.molbiopara.2018.04.003.

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