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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 *Pysera1* 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 exclusively 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*.

Keywords

Rodent malaria; *Plasmodium yoelii* ; Genome modifications; Cas9; knockin

1. Introduction

Malaria remains the most serious infectious disease worldwide. To uncover the molecular targets for the future development of better anti-malaria medicine and vaccine, new technologies and methods are still urgently needed for studying the biology of malaria parasites and mechanism of malaria disease and pathology [1, 2]. CRISPR/Cas9 (clustered regularly interspaced short palindromic repeats /CRISPR-associated protein 9), a powerful genome editing technology, has been recently successfully adapted for genome modification

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at

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 DSBs are repaired dominantly by homologous recombination (HR) pathways over the error-prone nonhomologous endjoining (NHEJ) pathway in *Plasmodium* [8, 9], therefore the donor templates were needed for HR repair in all the CRISPR/Cas9-mediated genome editing practices. For editing the genome of *P. falciparum*, the Cas9/sgRNA cassette and donor template were delivered into parasites in two separate vectors with each plasmid carrying a different drug resistant gene as selection marker [3, 5, 10–12]. For the rodent malaria parasite *P. yoelii*, a single vector system was applied because limited independent selection markers were 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 DNA were included in the plasmid vector. So far, the *Streptococcus pyogenes*-derived Cas9 (SpCas9) is the only endonuclease used for CRISPR/Cas9-mediated gene editing in malaria parasites [3–5], although the *SpCas9* coding sequence is about 4200 bp in size [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]. We thus reasoned that endogenous and constitutive expressing Cas9 derived from the Cas9-knockin malaria parasite could increase the gene editing efficiency and facilitate the application for different goals, in which the genome editing would only require introduction of sgRNA and homologous template components, benefiting the plasmid construction procedures and plasmid delivering into the parasite.

In this study, we replaced the coding region of an endogenous gene *Pysera1* 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 exclusively 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*.

2. Materials and Methods

2.1. Plasmid construction

The procedure for generating constructs for gene deletion, tagging, and replacement was as described previously [4]. To construct pYCm vector for deleting the coding region of *Pysera1* gene (PY17X_0305400), we amplified 526bp 5’ untranslated region (UTR) upstream of translation start codon as the left homologous arm and a 500bp 3’UTR region following translation stop codon as right homologous arms using PCR primers listed in Table S1. One sgRNA was designed to target the site in the coding region to be deleted.

All the fragments were sequentially ligated into the pYCm vector by T4 connection. To construct pYCm vector for replacing the coding region of *Pysera1* gene with the *SpCas9* coding sequence, we amplified the full-length *SpCas9* coding region from the plasmid pYC, tagged with a quadruple Myc epitope (4Myc) C-terminally, and inserted between the left and right homologous arms of the pYCm vector used for deleting *Pysera1* construct. For gene editing purpose in the *PyCas9ki* parasite, we engineered the pYCm vector and removed the *SpCas9* coding region via mutagenesis, resulting in a new vector, which we designed pYCs. To construct pYCs vectors for deleting the *Pycdpk3* gene (PY17X_0410700) and *Pyctrp* gene (PY17X_0415800), and for tagging the *Pysep1* (PY17X_0526200) and *Pydhhc10* genes (PY17X_0946500), the procedures are similarly followed. All the primers and oligonucleotides used are listed in Table S1.

2.2. Malaria parasite and parasite transfection

The parasite 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 *P. yoelii* 17XNL parasite or the *PyCas9ki* (*P.yoelii* 17XNL *Cas9 knockin*) strain. The procedures for parasite transfection, Pyr selection, and cloning were as described previously [4]. Briefly, parasites were electroporated with purified plasmid DNA. Transfected parasites were immediately intravenously injected into a naive mouse. Pyr (6ug/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 for next-round genome modification, the parasites were applied for 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. The 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 using 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. 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. 20 μl of the ookinete cultures were suspended and mixed with an equal volume of Matrigel (BD, USA) on ice. The mixtures were transferred on a slide, sealed with nail varnish after adding a coverslip, and put at 22°C for 30 min before the microscopy analysis. After finding an eye field containing ookinetes under microscopy, 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, Fuzhou, Fujian, P. R. 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, 40 female *Anopheles stephensi* mosquitoes were allowed to feed to one anaesthetized infected mice that carried comparable numbers of gametocytes as determined by Giemsa staining for 20 min. Twenty mosquitos were dissected day 7 post-infection, and oocysts in the midguts were counted. Salivary glands were isolated from 20–25 dissected mosquitos 14 days post-infection, and sporozoites were collected and counted using a hemocytometer under the microscopy.

2.8. Immunofluorescence assay

Parasite samples are harvested by centrifuging for 3 min at 2,000 rpm, washed twice with PBS, and resuspended 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 incubated with primary antibodies in 5% BSA in PBS for 12 h at 4°C, washed with PBS three times, and incubated with fluorescence conjugated secondary antibodies for 1 h. Subsequently, the cells were washed three times with PBS, and stained with Hoechst33342 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 microscopy with a 100× oil objective. Results were obtained from three independent experiments.

2.9. Western blotting

Parasite protein extraction was performed using RIPA lysis buffer (50mM Tris (pH 7.4), 150mM NaCl, 1%Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 2mM sodium pyrophosphate, 25mM β-glycerophosphate, 1mM EDTA, 1mM Na_3VO_4, 0.5 μg/ml leupeptin) plus 1× complete protease inhibitor cocktail and 1mM PMSF. After ultrasonic, the sample was incubated on ice for 30 min, and centrifuged at 12,000 g for 10 min at 4°C. The supernatant was collected, mixed with 5× protein loading buffer. To completely denature the proteins, the samples were treated at 95°C for 5 min and store at −20°C for next analysis. About 40 μg total protein was loaded and separated in 4%−9% SDS-PAGE. Proteins were transferred to a 0.22 μm PVDF membrane by wet transfer blotting for 1 h, blocked with 5% skim milk for 1 h, and probed with the specific antibodies. The proteins were detected using an enhanced chemiluminescent substrate kit (ECL) and visualized by the film at dark room.

2.8. Software

To search a potent sequence targeting by Cas9/sgRNA in the genone, we used the online program, Eukaryotic Pathogen CRISPR guide RNA/DNA Design Tool (http://grna.ctegd.uga.edu). To quantify the fluorescent signal in the fluorescence microscopy images, we used the Image J (https://imagej.nih.gov/ij). To record the ookinete's gliding route and calculate the motility speed, we used the Image J for the imaging processing and computing. For statistical analysis, we used the GraphPad Prism5 (https://www.graphpad.com).

3. Results

3.1. Generation of Cas9 knockin transgenic parasite

Because the *Streptococcus pyogenes Cas9* (*Spcas9*) gene is a 4200 bp coding sequence in size, it is a challenging to incorporate Cas9 expression cassettes with necessary genetic elements, including promoters and polyadenlyation sequences, into the genome of malaria parasite. Alternatively, we attempt to replace the coding region of specific endogenous gene with *cas9* coding sequence in the genome, and achieve expression of Cas9 protein driven by the regulatory sequence (promoter) of endogenous gene (Fig 1A). The parasite gene encoding a serine protease (*P. yoelii sera1, Pysera1*) has been shown to be nonessential for the parasite growth in the blood stage [4, 17]. To test the feasibility that *Pysera1* serves as a safe harbor for *cas9* integration and transcription in the transgenic parasite, we disrupted the *Pysera1* gene in the *P. yoelii* 17XNL parasite using CRISPR/Cas9 methods described previously [13], and obtained two parasite knockout clones (Δ*Pysera1c1* and Δ*Pysera1c2*) with deletion of the whole coding region (Fig S1A,S1B). Both Δ*Pysera1c1* and Δ*Pysera1c2* parasite clones display 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 *Pysera1* 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 *Pysera1* (0.5 kb of the 5'-flanking region and 0.5 kb of the 3'-flanking region) (Fig. 1A). Two parasite clones (*PyCas9kic1* and *PyCas9kic1*) with targeted modification in *Pysera1* locus were obtained and genotyped using diagnostic PCR (Fig. 1B). Corrected replacing *Pysera1* with *cas9* was further confirmed by RT-PCR analysis as the mRNA transcript of *cas9*, but not endogenous *Pysera1*, was specifically detected in the red blood stage of *PyCas9ki* parasites (Fig. 1C). Furthermore, we detected the expression of both Cas9::4Myc protein from integrated locus and 3Flag::Cas9 protein derived from the episomal pYCm plasmids using western blotting analysis (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 treating with 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 endogenous Cas9 expression on the parasite development and differentiation, we performed detailed analysis to compare the progression in 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 infection of mice with sporozoites compared with the results for WT, suggesting normal progression of *PyCas9ki* parasites during life cycle.

3.2. Specific 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, trophozoite, and schizont using anti-Myc antibody by immunofluorescence assay (IFA) (Fig 2A), which is similar with previous reports [4, 18]. 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 *Pysera1* gene possesses the transcription activity specifically in the asexual blood stage 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).

3.3. Endogenous gene deletion in the PyCas9ki parasite

Because only sgRNA cassette and homologous DNA template are needed to be introduced exogenously into the cell for CRISPR/cas9-mediated genome editing in the *PyCas9ki* parasite constitutively expressing Cas9 protein, we engineered the pYCm vector and removed the *SpCas9* coding region via mutagenesis, resulting in a new plasmid vector with a smaller size, which we designed pYCs (Fig. S3). To test whether the Cas9 protein expressed endogenously could be applied for CRISPR/Cas9-medifated gene modification, we attempted to deleted two genes (*Pyctrp* and *Pycdpk3*) 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, respectively, and absence of oocysts in the mosquito after infection [13, 19–22]. We constructed a plasmid pYCs-cdpk3 containing a 46-bp tag DNA (for PCR

primers) flanked by two homologous regions of *Pycdpk3* (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 *Pycdpk3*, 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* strain, 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 cloned parasites with disrupted *Pycdpk3* gene were obtained and confirmed by PCR genotyping (Fig. 3D). Using the same method, we successfully disrupted the *Pycdpk3* 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*Pycdpk3* and Δ*Pyctrp*) displayed normal asexual growth and gametocyte formation in the mouse (data not shown), and comparable conversion rate to mature ookinetes (Fig. 3G), but severe defect in ookinete gliding motility *in vitro* (Fig. 3F), which is consistent with the phenotype of *cdpk3* KO 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 localization and protein interaction. To test this application in the *PyCas9ki* parasite, we built a construct (pYCs-Pysep1::6HA) containing a 498 bp C-terminal region of the *Pysep1* gene followed by a sextuple HA tag (6HA) and a 512-bp 3'-flanking region (3'-UTR) of the *Pysep1* gene (PY17X_0526200) (Fig 4A). The *Pysep1* gene encodes an early transcribed membrane protein locating at the parasitophorous vacuole membrane (PVM) [23]. We detected the integration of both donor templates into the 3' end of *Pysep1* 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 [23]. In addition, we attempted to knock in a 4Myc epitope tag in another genes *Pydhhc10* (PY17X_0946500) (Fig 4E), the orthologue of which encodes a S-acyl-transferase expressing only in gametocytes and ookinetes of *P. berghei* [24]. Targeted knockin of the 4Myc tag in the 3' end of *Pydhhc10* coding region was detected in the two parasite clones using genotypic PCR (Fig 4F). Furthermore, the expression of recombinant DHHC10::4Myc protein was detected in ookinetes of the two parasite clones in the analysis of 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 [24]. Together, these results indicate that tagging endogenous genes could be achieved via CRISPR/Cas9 in the *PyCas9ki* parasite.

4. Discussions

In this study, we present the generation of a transgenic rodent malaria parasite *P. yoelii* 17XNL strain *PyCas9ki*, where the CRPSPR associated protein Cas9 encoding gene was knocked in the endogenous *Pysera1* gene locus and its expression is driven by the endogenous promoter of *Pysera1* gene. The resulted *PyCas9ki* parasite displays normal progression during the whole life cycle and possesses the Cas9 protein expression exclusively in asexual blood stage. Furthermore, we demonstrate that both gene disruption and gene tagging, the most commonly used gene editing for gene function study, could be efficiently achieved in this *PyCas9ki* parasite conjugation with the pYCs vector.

So far, several CRISPR/Cas9-based applications have been described for the genome modification in *P. falciparum* and *P. yoelii* [3–5, 10–13]. In these Cas9 vector-based practice, the transcription and expression of exogenous Cas9 in the parasite was driven by the promoters, such as *P. falciparum hsp86* promoter and *P. yoelii* eef1aa promoters, which possess the transcription activity for the whole parasite life cycle. Currently, the asexual blood stage is the only stage window for genetically manipulation in malaria parasites [2]. An idea design for exogenous Cas9 expression would be the asexual blood stage specific expression of the Cas9 because of the existence of the potent activity of caused by off-target effect of the endonuclease Cas9 [25, 26]. Fortunately, the endogenous Cas9 protein was expressed exclusively in the asexual blood stage of the resulted *PyCas9ki* parasite, including the ring, troph, and schizont (Fig 2A), excluding the possibility of the Cas9 derived off-target effect in the other stages of life cycle.

In our previous studies, we developed a CRISPR/Cas9-based single vector system pYC/pYCm to successfully modify *P. yoelii* genome, including gene deletion, gene tagging, and nucleotide replacement [4, 13]. In this one-vector plasmid pYCm design (Fig S3), all components, including the Cas9 and sgRNA expression cassettes, the multiple cloning site for insertion of donor template DNA, and the fused selectable markers (*hdhfr* and *yfcu* for sequential positive and negative selection) were included. We engineered an updated plasmid pYCs derived from the original pYCm, where the Cas9 encoding region was removed, and the sgRNA cassettes, the multiple cloning site, and the *hdhfr*/*yfcu* selection marker were sustained (Fig S3). Compared with the size (10.0 kb) of pYCm plasmid, this new “Cas9-free” plasmid pYCs (5.7 kb) thus can permit an introduction of larger donor DNA sequences, which could be required in some certain types of genome editing in the future.

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 locus simultaneously in the genome of this *PyCas9ki* parasite. The use of *PyCas9ki* parasite in conjunction with the pYCs vector containing multiplex sgRNA cassesste and their responding donor templetes for homologous recombination repair, technically make it feasible to easily introduce multiple genetic lesions in the same genome. As multigene interactions play an important role in many biological processes of eukaryotic organisms, multiplex genetic perturbation using the *PyCas9ki* parasite will enables the interrogation of complex effects for the multiple-members gene family in the life cycle development and differentiation of malaria parasite.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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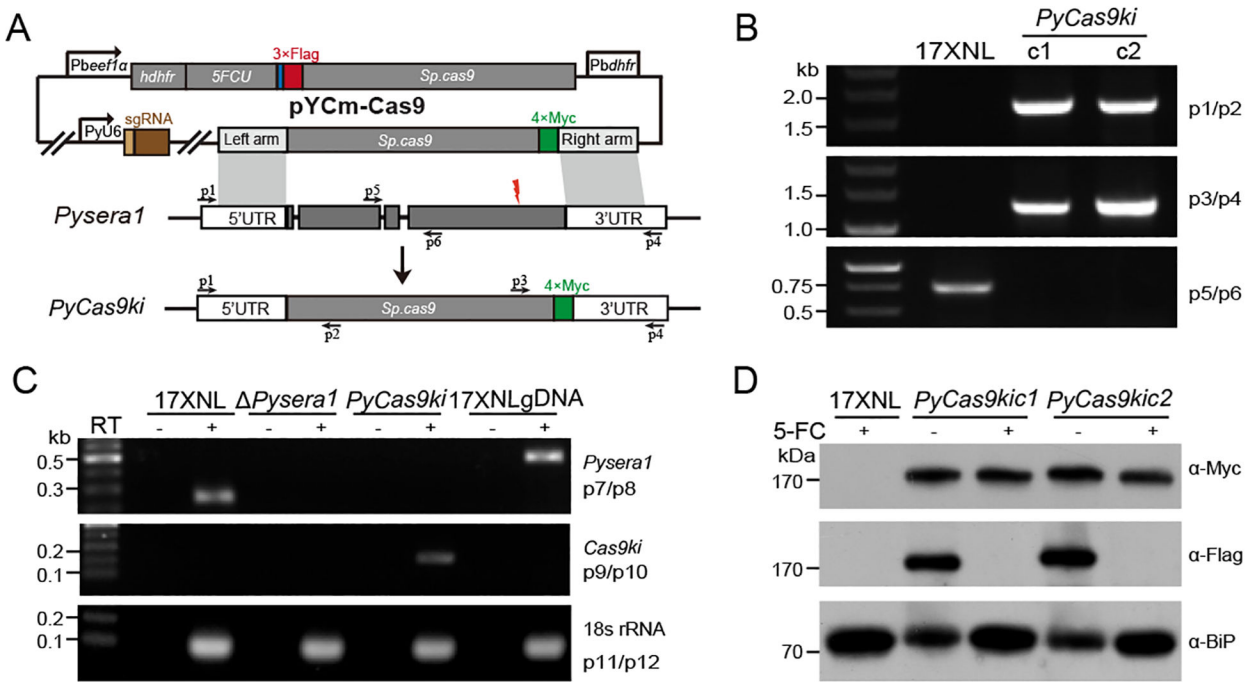

Fig. 1.

Generation of Cas9 knockin parasite of *Plasmodium yoelii*.

A. Schematic diagram of CRISPR/cas9 plasmid pYCm construct for replacing the endogenous *sera1* gene coding region with *cas9* gene in *Plasmodium yoelii*. The site for designed sgRNA recognition was indicated as red thunderbolt. Cas9 protein tagged with quadruple Myc epitope (4Myc, green) is for transgenic, and Cas9 protein tagged with triple Flag epitope (3Flag, Red) is for CRISPR/cas9 modification respectively.

B. Diagnostic PCR of two transgenic parasite clones (*PyCas9kic1* and *PyCas9kic1*) with targeted modification in *Pysera1* 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*Pysera1*, 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 and anti-Myc antibodies were used for detecting the expression of the 3Flag::Cas9 and 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.

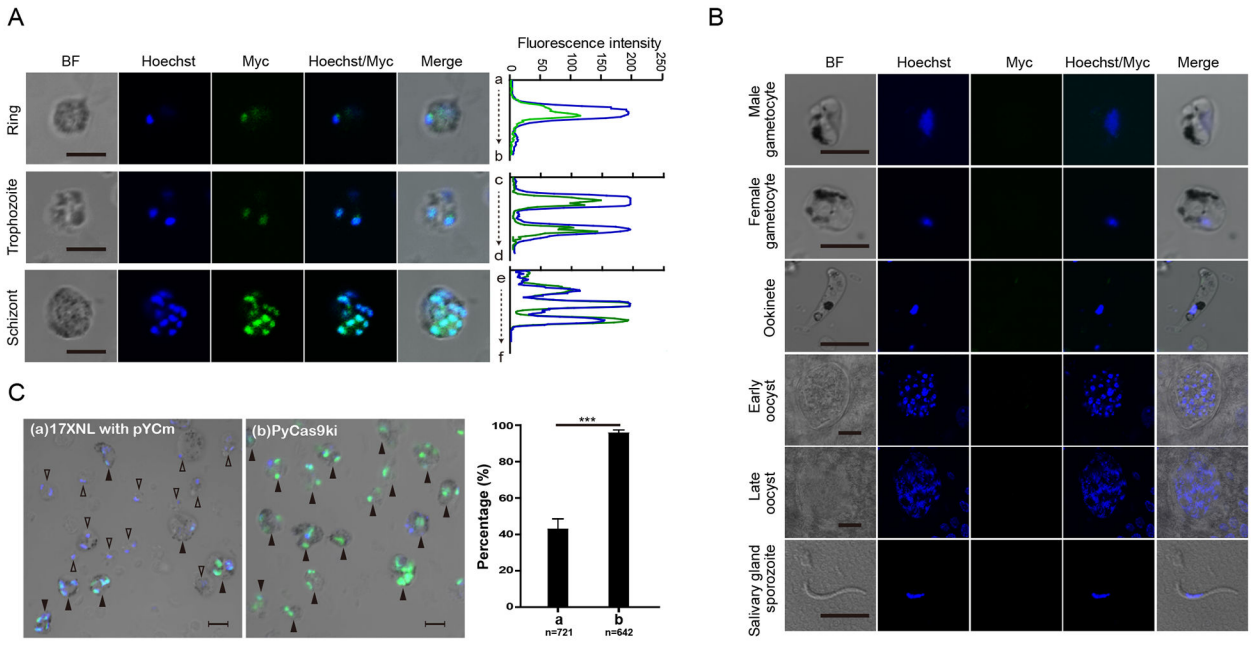

Fig. 2.

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

A. Immunofluorescence assay (IFA) of Cas9 protein expression in the asexual blood stage of *PyCas9ki* parasite, including ring, trophozoite, and scizhont. Nuclei were stained with Hoechst33342 (blue). Bar = 5 μm. Results are representative of three independent experiments. Co-localization analysis between Cas9 protein and nucleus was shown at the right pannel.

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

C. IFA analysis of Cas9 protein expression in the asexual blood stage of 17XNL parasite transfected with episomal pYCm and the *PyCas9ki* parasite. Parasites with Cas9 protein expressing and co-localizing with nuclei were indicated by solid triangle ▲, the parasites without Cas9 protein expression were indicated by empty triangle △. quantification results were shown in the right panel. Bar = 5 μm.

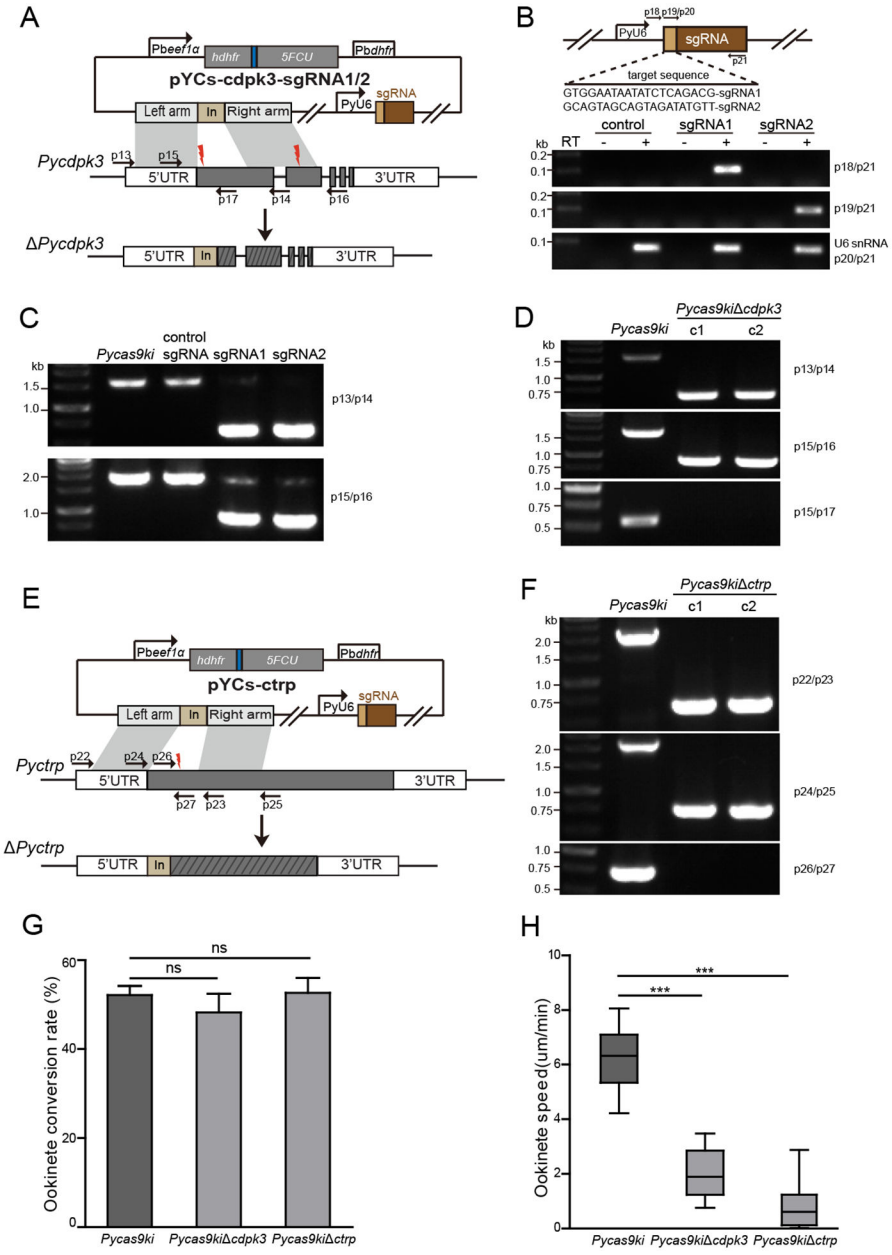

Fig. 3.

Deletion and mutant phenotype of endogenous *cdpk3* gene in the *PyCas9ki* parasite

A. Schematic diagram of pYCs construct for deleting the endogenous *cdpk3* gene. The site for designed sgRNA recognition was indicated as red thunderbolt.

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 as light orange box. sgRNA transcripts were detected by RT-PCR using specific primer pair (p19/p21 and p20/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 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. *In vitro* ookinete differentiation of the parental *PyCas9ki* and *PyCas9kiΔcdpk3* parasites.

F. Ookinete gliding motility of the parental *PyCas9ki* and *PyCas9kiΔcdpk3* parasite using Matrigel motility assay. At least 20 cultured ookinetes were measured over a 20 min period. Results are representative of two independent experiments.

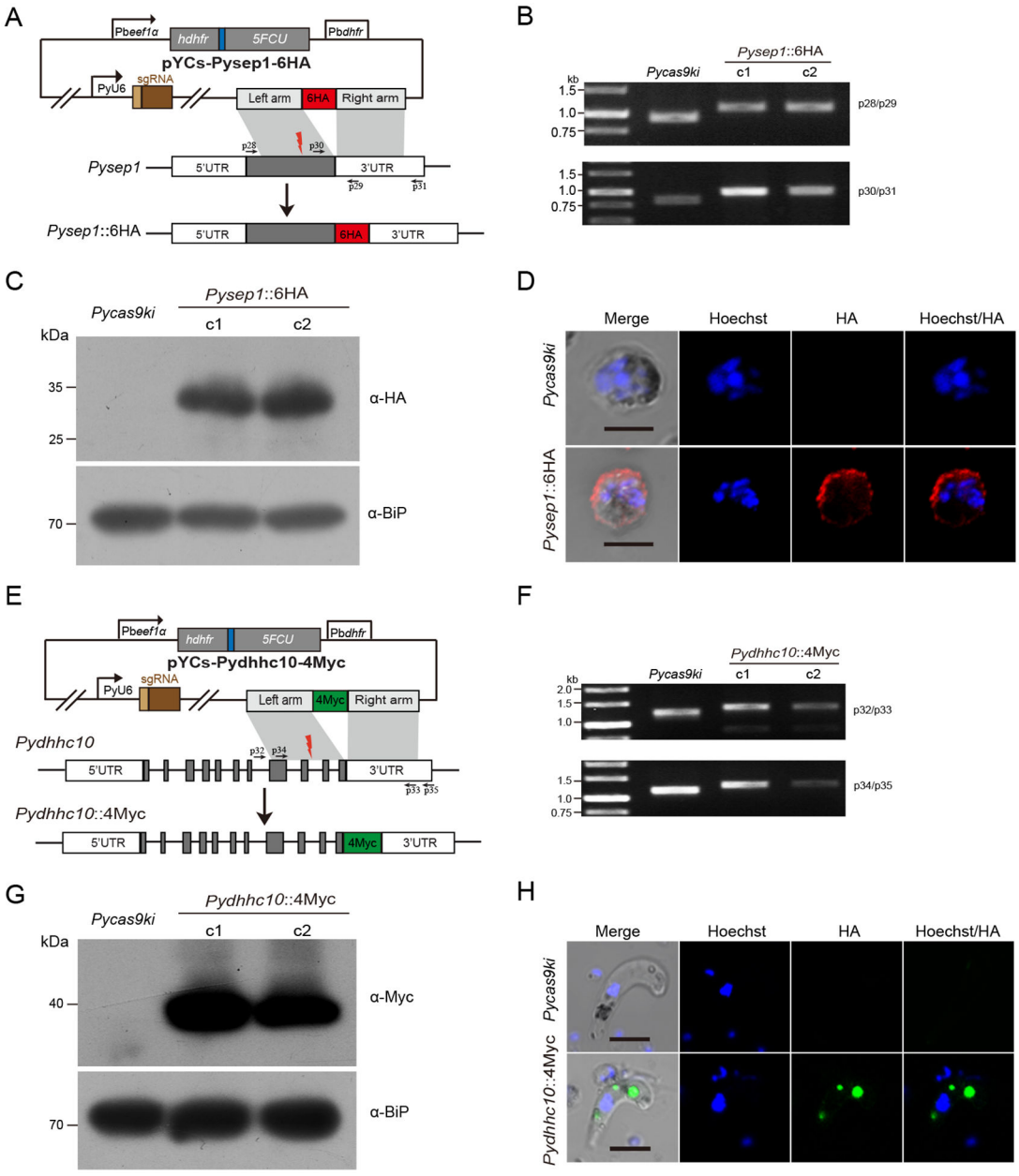

Fig. 4.

Tagging and protein expression of endogenous *sep1* and *dhhc10* genes in the *PyCas9ki* parasite

A. Schematic diagram of pYCs construct for tagging the *sep1* gene with 6HA. The site for designed sgRNA recognition was indicated as red thunderbolt.

B. PCR detection of the *PyCas9ki* parasite and two *PyCas9ki* derived clones with *sep1* gene tagging with 6HA C-terminally.

C. Western blotting analysis of 6HA tagged Sep1 protein expression in the asexual blood stage of the *PyCas9ki* parasite and two *PyCas9ki* derived clones. Anti-HA antibody was used for the detection. BiP protein serves as an internal control.

D. IFA analysis of 6HA tagged Sep1 protein expression in the asexual blood stage of the *PyCas9ki* parasite and one *PyCas9ki* derived clones. Nuclei were stained with Hoechst33342 (blue). Bar = 5 μm.

E. Schematic diagram of pYCs construct for tagging the *dhhc10* gene with 4Myc. The site for designed sgRNA recognition was indicated as red thunderbolt.

F. PCR detection of the *PyCas9ki* parasite and two *PyCas9ki* derived clones with *dhhc10* gene tagging with 4Myc C-terminally.

G. Western blotting analysis of the 4Myc tagged DHHC10 protein expression in the ookinetes of the *PyCas9ki* parasite and two *PyCas9ki* derived clones. Anti-Myc antibody was used for the detection. BiP protein serves as an internal control.

H. IFA analysis of the 4Myc tagged DHHC10 protein expression in the ookinetes of the *PyCas9ki* parasite and one *PyCas9ki* derived clones. Nuclei were stained with Hoechst33342 (blue). Bar = 5 μm.