Molecular & Biochemical Parasitology 241 (2021) 111346

Contents lists available at ScienceDirect

Molecular & Biochemical Parasitology

journal homepage: www.elsevier.com/locate/molbiopara

Generation of *Plasmodium yoelii* malaria parasite for conditional degradation of proteins

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ARTICLE INFO

Keywords:
Malaria parasite
Plasmodium yoelii
Protein degradation
AID

ABSTRACT

The auxin-inducible degron (AID) system is a robust chemical-genetic method for manipulating endogenous protein level by conditional proteasomal degradation *via* a small molecule. So far, this system has not been adapted in the *P. yoelii*, an important and widely used *Plasmodium* rodent parasite model for malaria biology. Here, using the CRISPR/Cas9 genome editing method, we generated two marker-free transgenic *P. yoelii* parasite lines (*eef1a-Tir1* and *soap-Tir1*) stably expressing the *Oryza sativa* gene *tir1* under the promoters of *eef1a* and *soap* respectively. These two lines develop normally during the parasite life cycle. In these backgrounds, we used the CRISPR/Cas9 method to tag two genes (*cdc50c* and *fbxo1*) with the AID motif and interrogate the expression of these two proteins with auxin. The *eef1a-Tir1* line allows efficient degradation of the AID-tagged endogenous protein in the asexual schizont and sexual gametocyte stages, while the *soap-Tir1* line allows protein degradation in the ookinetes. These two lines will be a useful resource for studying the *Plasmodium* parasite biology based on the *P. yoelii*.

1. Introduction

Malaria remains one of the most serious infectious diseases worldwide. Rodent malaria parasites *Plasmodium berghei* and *Plasmodium yoelii* have been widely used as model systems to study malaria pathology and parasite biology. These species possess many biological features similar to those of the human malaria parasites in vertebrate hosts and mosquito vectors during life cycle. Recent years has witnessed great progress in developing research tools, in particular the CRISPR/Cas9 genome editing technology [1–3], which enables more efficient interrogation of gene function in malaria parasites. Because asexual blood stage of the parasites is the only time window suitable for DNA transfection, the lack of robust method hampers the functional investigation of genes essential for asexual blood stage development of *Plasmodium* parasites [4]. In the past decade or so, the conditional gene knockout approach was demonstrated in both the *P. falciparum* and *P. berghei*, wherein the genetic alteration at the genomic DNA level is irreversible and thereby the functional analysis is allowed only at its first point of protein action [5–7]. Methods to inducible control transcription have also been reported in the *P. falciparum* and *P. berghei*, but suffer from slow onset kinetics and were tested only for asexual blood stage [8–11]. Different from the strategies targeting in the DNA or mRNA, manipulation of protein levels by chemical-genetic strategies is often more advantageous in inducibility, reversibility, and specificity. Two such regulatory systems composing of specific pair of ligands and associated protein binding domains have been developed in the *P. falciparum* to regulate protein stability using either FKBP protein destabilization domain or dihydrofolate reductase destabilization domain [12–14].

Plants evolved a unique system in which the plant hormone auxin induces rapid proteasomal degradation of certain proteins by a specific E3 ubiquitin ligase [15]. Kohei

Nishimura et al. creatively utilized the auxin-inducible degron (AID) in the non-plant cells and achieved conditional degradation of proteins of interest (POI) *via* a small molecule [16]. The system relies on the evolutionarily conserved eukaryotic SCF ubiquitin ligase complex composing of the Skp1, Cullin1, Rbx1, and F box protein, the last of which recruits protein substrates for degradation. Recently, the AID system has also been successfully adapted in the apicomplexan parasites, including *P. berghei* [17,18] and *Toxoplasma gondii* [19]. To implement this system, only two transgenic components are needed: a plant auxin receptor called transport inhibitor response 1 (TIR1) and a POI tagged with an AID. In these engineered parasite lines stably expressing the

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https://doi.org/10.1016/j.molbiopara.2020.111346
Received 22 August 2020; Received in revised form 30 November 2020; Accepted 2 December 2020
Available online 8 December 2020

plant TIR1 [18,19], the auxin functions as a molecular glue promoting specific interaction between the ubiquitin ligase complex and AID-tagged POI, which triggers proteasomal degradation of the latter.

So far, the application of AID-mediated protein degradation has not been reported in the *P. yoelii*. Inability to manipulate the protein essential for parasite asexual blood stage development prompts us to develop an AID system in the *P. yoelii*. Here, using the CRISPR/Cas9 method, we generated two marker-free transgenic parasite lines *eef1a-Tir1* and *soap-Tir1*, which stably express the *Oryza sativa* gene *tir1* under the promoters of *eef1a* and *soap* genes respectively. These two lines develop normally during the life cycle. While the *eef1a-Tir1* line allows efficient degradation of the engineered endogenous protein containing an AID motif in the schizonts and gametocytes, the *soap-Tir1* line enables protein degradation in the ookinetes.

2. Materials and methods

2.1. Animal use and ethics statement

The *P. yoelii* 17XNL strain parasite was used in this study and 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.

2.2. Plasmid construction

The CRISPR/Cas9 plasmid pYCm was used to edit the parasite genome [2,20]. To generate the plasmid for deleting the gene *p230p* (PY17X_0306600), a 973 bp of the 5′ untranslational region (UTR) upstream the initiation codon and an 857 bp of the 3′UTR following the translation stop codon were amplified as the homologous left and right arm, respectively. The left and right arms were inserted into the pYCm plasmid. Eight single guide RNAs (sgRNAs) were designed to target the coding region of the *p230p* gene. To generate the plasmid for replacing the coding region of *p230p* gene with the *Tir1* expression cassette, the coding sequence of *Tir1* was amplified from the *Oryza sativa* genome, tagged with a Flag epitope sequence, and put under the control of both the 5′UTR (promoter) of *eef1a* (551 bp) and the 3′UTR of the *dhfr* (456 bp). The *Tir1* expression cassette was inserted between the left and right homologous arms in the pYCm plasmid. To generate the plasmid for tagging the *cdc50c* (PY17X_0514500) with an AID degron and sextuple HA fusing epitope (AID::6HA), the C-terminal 518 bp of coding region and 529 bp of the 3′UTR of the *cdc50c* gene were amplified as the homologous left and right arm, respectively. One sgRNA was designed to target the C-terminal coding region of *cdc50c* genes. To generate the plasmid for tagging the *fbxo1* (PY17X_ 1120000) with the AID::6HA, the C-terminal 498 bp of coding region and 521 bp of the 3′UTR of the *fbxo1* gene were amplified as the homologous left and right arm, respectively. One sgRNA was designed to target the C-terminal coding region of *fbxo1* genes. All primers and oligonucleotides used were listed in the Table S1.

2.3. Parasite transfection and genotyping

The procedures for parasite transfection, pyrimethamine (pyr) selection, and single cloning were as described previously [2,20]. Purified parasites were electroporated with 5 μg circular plasmid DNA, immediately injected *i.v.* into a naïve mouse, and subjected to selection with pyr provided in drinking water at a concentration of 6 mg/L from day 2 after electroporation. Pyr resistant parasites usually appear 5–6 days after drug selection. Parasite genomic DNAs from infected mouse blood were isolated and used for PCR genotyping. Correct 5′ and 3′ integrations were confirmed by PCR using the specific pairs of primers in the Table S1.

2.4. Negative selection with 5-Fluorocytosine

To remove the plasmids within the transfected parasites after prior pyr drug selection, parasites were subjected to negative selection with 5-Fluorocytosine (5-FC, Sigma Aldrich, F6627) as described previously [20]. 5-FC 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.

2.5. Gametocyte induction and purification

Gametocyte induction and purification was performed using a modified protocol from [21,22]. Briefly, mice were treated by intraperitoneal injection of phenylhydrazine (PHZ, 80 μg/g mouse body weight) for hemolysis to enhance gametocytogenesis. Three days post treatment, mice were infected with 2.0×10^6 parasites by tail vein injection. Peaks of gametocytemia were usually observed three days after post infection. Mouse blood containing more than 5% gametocytemia were collected and loaded for centrifugation on a Nycodenz cushion (27.6 % w/v Nycodenz in 5.0 mM Tris-HCl, 3.0 mM KCl, 0.3 mM EDTA). Gametocytes were harvested from the interphase and washed three times with gametocyte maintenance buffer (137 mM NaCl, 4 mM KCl, 1 mM $CaCl_2$, 20 mM glucose, 20 mM HEPES, 4 mM $NaHCO_3$) for further use.

2.6. Exflagellation assay

Two and a half microliters of mouse tail blood with 4–6 % gametocytemia were added to 100 μl exflagellation medium (RPMI 1640, 10 % fetal calf serum/FCS, 100 μM xanthurenic acid/XA, and pH 7.4) containing 1 μl of 200 unit/mL heparin for 10 min at 22 °C. For male gametocyte activation, the exflagellation centers (EC) and total RBC were counted in a hemocytometer under light microscope. The percentage of RBCs containing male gametocytes was calculated from Giemsa-stained smears, and the number of ECs per 100 male gametocytes was calculated as male gamete formation rate.

2.7. In vitro ookinete culture

Ookinetes were prepared in *in vitro* culture according to the procedure [23]. Briefly, 200 μL of mouse blood containing gametocytes was mixed with 1 mL ookinete culture medium (RPMI 1640, 10 % FCS, 100 μM XA, 25 mM HEPES, and pH 8.0) and incubated at 22 °C to allow gametogenesis, fertilization, and ookinete differentiation. The zygotes, retort, and mature ookinetes were taken from culture at 2, 6, and 12 h, respectively.

2.8. Mosquito transmission of parasite

After checking the gametocytemia in the infected mice by Giemsa staining, 40–50 female *Anopheles stephensi* mosquitoes were allowed to feed on one anaesthetized mice carrying gametocytes for 20 min. 20–25 mosquitoes were dissected at day 7 post-infection, and oocysts in the midgut were counted. Salivary glands were isolated from 20 to 25 dissected mosquitoes at day 14 post-infection, and sporozoites were collected and counted using a hemocytometer under the microscopy.

2.9. IAA treatment and protein depletion

A stock solution of 250 mM IAA (Indole 3-acetic acid, Sigma Aldrich, I2886) was prepared using the 100 % EtOH. Mock treatment includes an equivalent volume of 100 % EtOH. IAA was dissolved in the schizont media (80 % RPMI1640, 20 % FCS, penicillin and streptomycin), in the

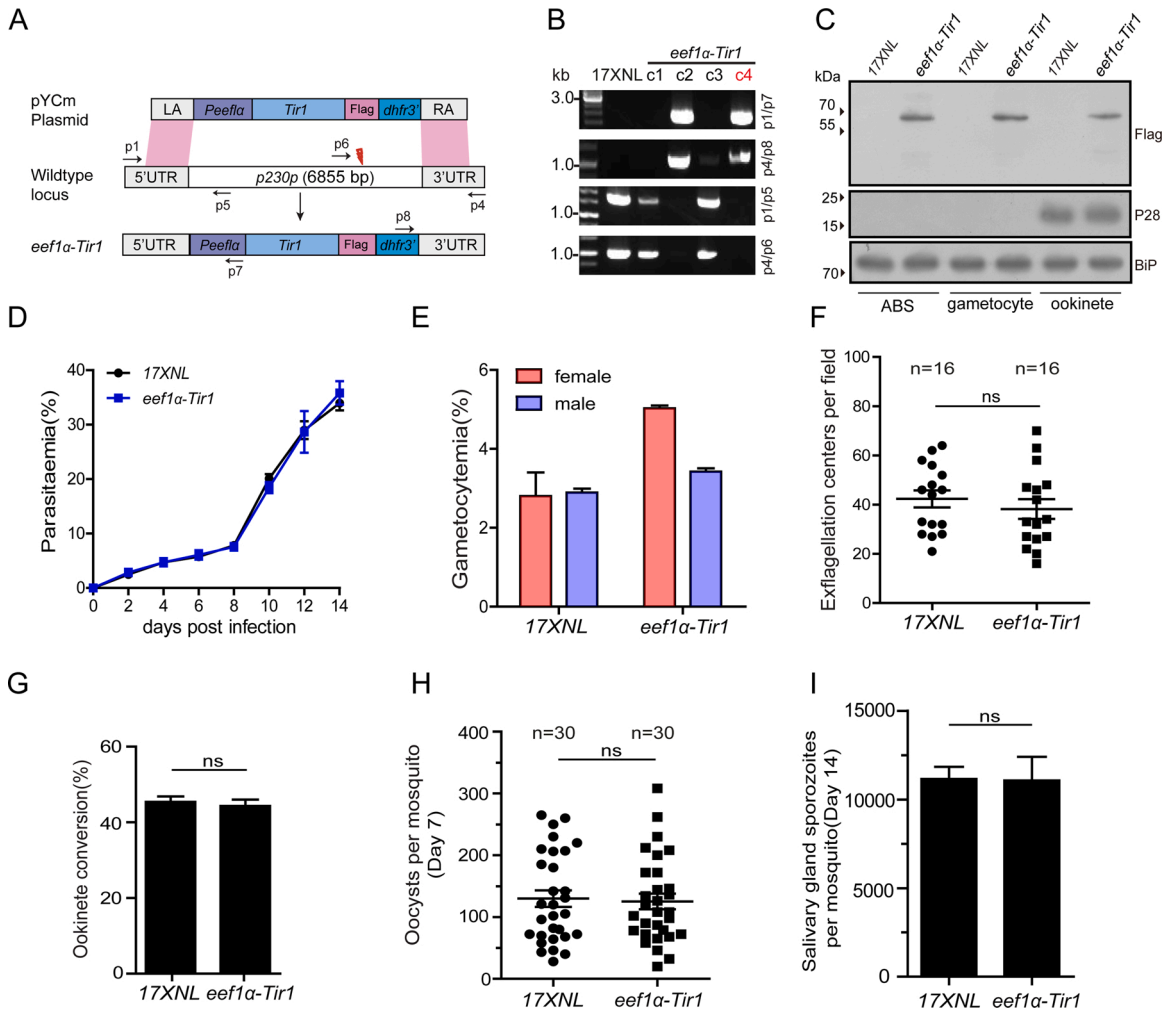

Fig. 1. Generation and characterization of *P. yoelii* parasite line *eef1a-Tir1* stably expressing the *Oryza sativa tir1* gene under the promoters of *eef1a*.
A. Diagram of CRISPR/cas9 mediated replacement of the coding region of the *p230p* gene with a *Tir1* expressing cassette. The *Tir1::Flag* was driven by the promoter of *eef1a* and the 3′UTR of the *dhfr* gene. "p" indicates the positions of PCR primers for genotyping analysis. The red thunderbolt indicates sgRNA recognition site.
B. Genotyping of the transgenic *eef1a-Tir1* line. Four parasite clones (c) were obtained after limiting dilution, and the clone indicated with red letter was used for further analysis.
C. Western blot analysis of transgenic Flag-tagged Tir1 protein in the cell lysates of parasite asexual blood stage (ABS), gametocytes, and ookinetes. BiP and P28 were the loading controls.
D. Parasite asexual blood stage proliferation in mice. The results were means from three mice in each group.
E. Gametocytes formation in mice. Female and male gametocytes were counted microscopically from mouse blood smear after Giemsa stain at day 3 post infection.
F. *In vitro* exflagellation centers of male gametocytes indicating male gamete formation. n is the number of microscopy fields observed in each group. Two-tailed unpaired Student's *t*-test.
G. *In vitro* ookinete formation. Mean ± SEM from three experiment, and two-tailed unpaired Student's *t*-test.
H. Midgut oocyst formation in the mosquitoes at day 7 post blood feeding. n is the number of mosquitoes dissected in each group. Mann–Whitney test applied.
I. Salivary gland sporozoite formation in the mosquitoes at day 14 post blood feeding. Mean ± SEM from three experiments, and two-tailed unpaired Student's *t*-test.

gametocyte maintenance buffer (GMB, containing 0.1 % BSA), or in the ookinete culture medium (RPMI 1640, 10 % FCS, 100 μM XA, 25 mM HEPES, and pH 8.0) for the final concentration indicated. To assess the degradation kinetics of targeting proteins, the asexual blood stage parasites, schizonts, gametocytes, and ookinete culture were incubated with 1 or 4 mM IAA at 37℃ for the time indicated. Times required for complete protein degradation are supposed to vary based on protein abundance, localization and stability. After incubation, the parasites were collected for protein expression analysis.

2.10. Western blot

Total proteins extracted from parasites of different stages were separated on 4.5 % SDS-polyacrylamide gels and transferred to PVDF membranes (Millipore). The blot was incubated with blocking buffer (PBS with 3% bovine serum albumin) and then incubated with rabbit anti-HA (1:1,000, Cell Signaling Technology, RRID: AB_1549585) or rabbit anti-Flag (1:1,000, Sigma Aldrich, RRID:AB_796202). Antiserum against BiP, P28, and Hep17 were previously prepared in the lab [24]. Antigen for P28: VTVDTICTNGKLIQMTSHLECKCIPGYGLKNENTCEKIVKCDQLDNI NKVCGEYAICGNQATLGLEKALVCSCVNGYLLSQNIC KPTKCFNYECPSGKCILDPNNPNNPTCSCDIGKIMQNGKCTGVGQAK-CALKCKATEECKLVGTYYECISKNPAPGTGSGTGSGTGS GTGSGTGTPANSSI. Antigen for BiP: DNYIQS MKATVEDKDKLADKIEKEDKDTILNAIKEAEDWLNNNSNADSEALKQKL KDVEAICQPIIVKLYGQPGAASPPPGDEDVDSDEL. Antigen for Hep17: MKINIASIIFIIFSLCLVNDAYGKNKYGKNGKYGSQNVIKKHGEPVINVQ DLISDMVRKEEEIVKLTKNKKSLRKINVALATALSVVSAILLGGAGLVM YNTEKGRRPFQIGKSKKGGSAMARDSSFPMNEESPLGFSPEEMEAVASK-FRESMLKDGVPAPSNTPNVQN. Antiserum against α-enolase was

prepared by immunization of rabbit with synthetic peptide (KTYDLDFKTPNNDK, rabbit, 1:1000). HRP-conjugated goat anti-rabbit antibody (1:5000, Sigma) was incubated with PVDF membranes for ECL detection. Signal intensity of protein bands in western blots was quantified using the ImageJ software.

2.11. Immunofluorescence assay

Schizonts and ookinetes were fixed with 4% paraformaldehyde for 15 min and rinsed with PBS three times. After permeabilized with 0.1 % Triton X-100 for 10 min, rinsed with PBS twice, and incubated with 5% BSA for 1 h, the parasites were incubated with the rabbit anti-HA primary antibody (1:1,000, Cell Signaling Technology, RRID:AB_1549585) or antiserum against P28 (1:1000) or GAP45 (1:1000) overnight at 4°C, rinsed with PBS three times, and incubated with fluorescent conjugated secondary antibody (Alexa 555 labeled goat anti-mouse and Alexa 488 labeled goat anti-rabbit IgG) for 1 h. After three washes with PBS, they were stained with the DNA dye Hoechst 33342 for 10 min and mounted on glass slides using mounting medium. All images were captured and processed using identical settings on a Zeiss LSM 780 confocal microscope. The fluorescent intensity of proteins was quantified using the ImageJ software.

3. Results and discussions

To generate a *P. yoelii* parasite line stably expressing the *Oryza sativa* gene *tir1*, we chose to insert a *tir1* expressing cassette into the locus of a non-essential gene. Gene *p230p* may fit such criteria because it was reported to be non-essential for life cycle development of *P. berghei* [18,25]. To test if this gene (PY17X_0306600) is essential for the life cycle of the *P. yoelii*, we disrupted *p230p* in the 17XNL strain using the CRISPR/Cas9 technology [2,20]. The obtained mutant clone is referred to as Δ*p230p* (Fig. S1A and B). The Δ*p230p* parasites displayed normal during the life cycle (Fig. S1C-G), indicating that *p230p* is dispensable for the *P. yoelii*. Therefore, we inserted the *tir1* expressing cassette into the *p230p* locus using the CRISPR/Cas9 method. The 5′-UTR of two genes *hsp70* (1755 bp) and *eef1a* (551 bp) as well as the 3′-UTR of gene *dhfr* (456 bp) were selected to drive the *Tir1::Flag* expression (Fig. 1A). While we readily obtained an *eef1a-Tir1* line (Fig. 1B), we failed to obtain any *hsp70-Tir1* line after several independent attempts using eight different sgRNAs. After removing the episome plasmids in the *eef1a-Tir1* by negative selection using 5-FC [20], the expression of Tir1::Flag fusion protein was detected in the asexual blood stage, gametocytes, and ookinetes by immunoblot (Fig. 1C). To evaluate the effect of Tir1 expression on the parasite development, we compared the parasite development during life cycle between the 17XNL and *eef1a-Tir1*. The *eef1a-Tir1* parasites displayed normal asexual proliferation and gametocytes formation in mice (Fig. 1D and E), and male gametocyte activation *in vitro* (Fig. 1F). In addition, formation of midgut oocysts (Fig. 1H), salivary gland sporozoites in the mosquitoes (Fig. 1I), and sporozoite infectivity (data not shown) are comparable between the *eef1a-Tir1* parasites and the 17XNL controls. These data indicate that the ectopic Tir1 expression does not impair the parasite development.

Using the above described *eef1a-Tir1* line, we first test the feasibility of AID-mediated degradation of the overexpressed exogenous protein. Towards this end, we made a construct to overexpress AID peptide tagged with a sextuple HA (6HA) (Fig. S2A), which is driven by the promoter (1805 bp) of *clag1* (PY17X_1402200) and the 3′-UTR of *dhfr*. Proper expression of AID-6HA peptide is confirmed by immunoblotting in the parasites at the asexual blood stage (Fig. S2A). Next, we tested if the AID::6HA peptide could be depleted in an auxin-dependent manner. Addition of auxin (1 mM) to the parasites prepared at asexual blood stage containing a mixture of the rings, trophozoites, and schizonts promoted rapid depletion of AID::6HA (Fig. S2B). However, a low

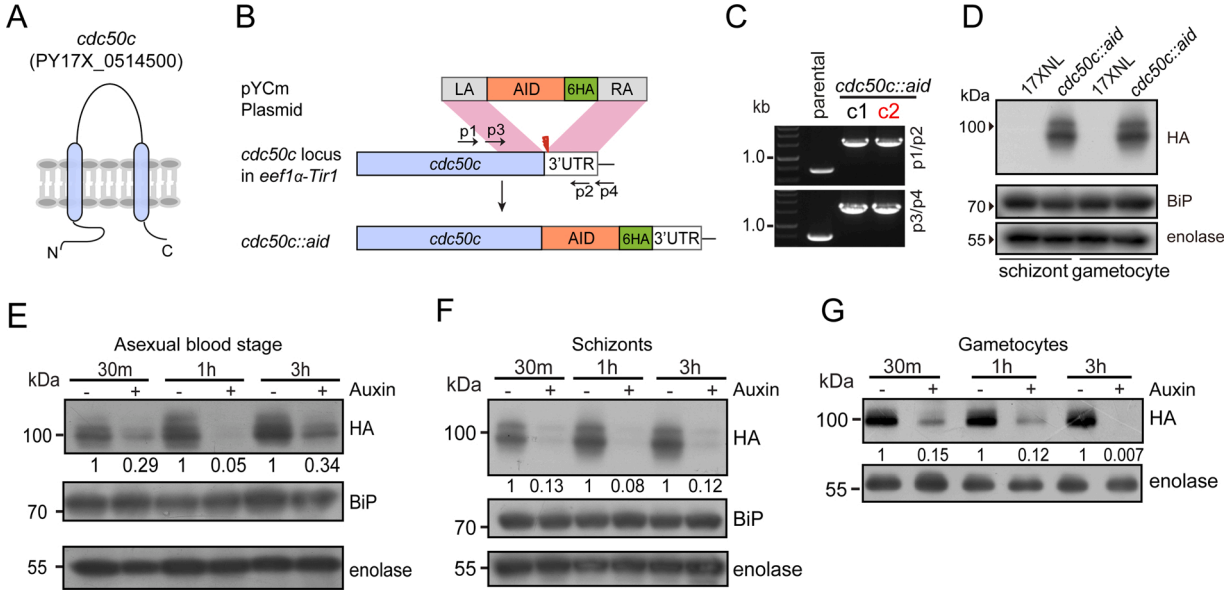

Fig. 2. Conditional depletion of the CDC50C protein in the *eef1a-Tir1* background.
A. Diagram of the *P. yoelii* CDC50C protein. CDC50C is a putative membrane protein with two transmembrane segments and a large exocytosolic loop.
B. Diagram of CRISPR/cas9 mediated tagging of the *cdc50c* gene with a sequence encoding the AID::6HA motif in the *eef1a-Tir1* line. "p" indicates PCR primers. Red thunderbolt indicates sgRNA recognition site.
C. Genotyping of the resulting strain *eef1a-Tir1/cdc50c::aid*. Two parasite clones (c) were obtained, and the clone indicated with red letter is used for further analysis.
D. Western blot analysis of the HA-tagged CDC50C protein expression in the schizonts and gametocytes of *eef1a-Tir1/cdc50c::aid* parasite. BiP and enolase were the controls.
E. Efficient depletion of CDC50A-AID in the asexual blood stage parasites containing ring, trophozoite, and schizonts, as measured by western blot. The parasites were treated with 1 mM auxin for indicated time. BiP and enolase serve as the controls. The numbers are the relative intensities of protein band in blot.
F. Depletion of CDC50A-AID in the purified schizonts treated with 1 mM auxin. BiP and enolase serve as the controls.
G. Depletion of CDC50A-AID in the purified gametocytes treated with 1 mM auxin. Enolase serves as the control.

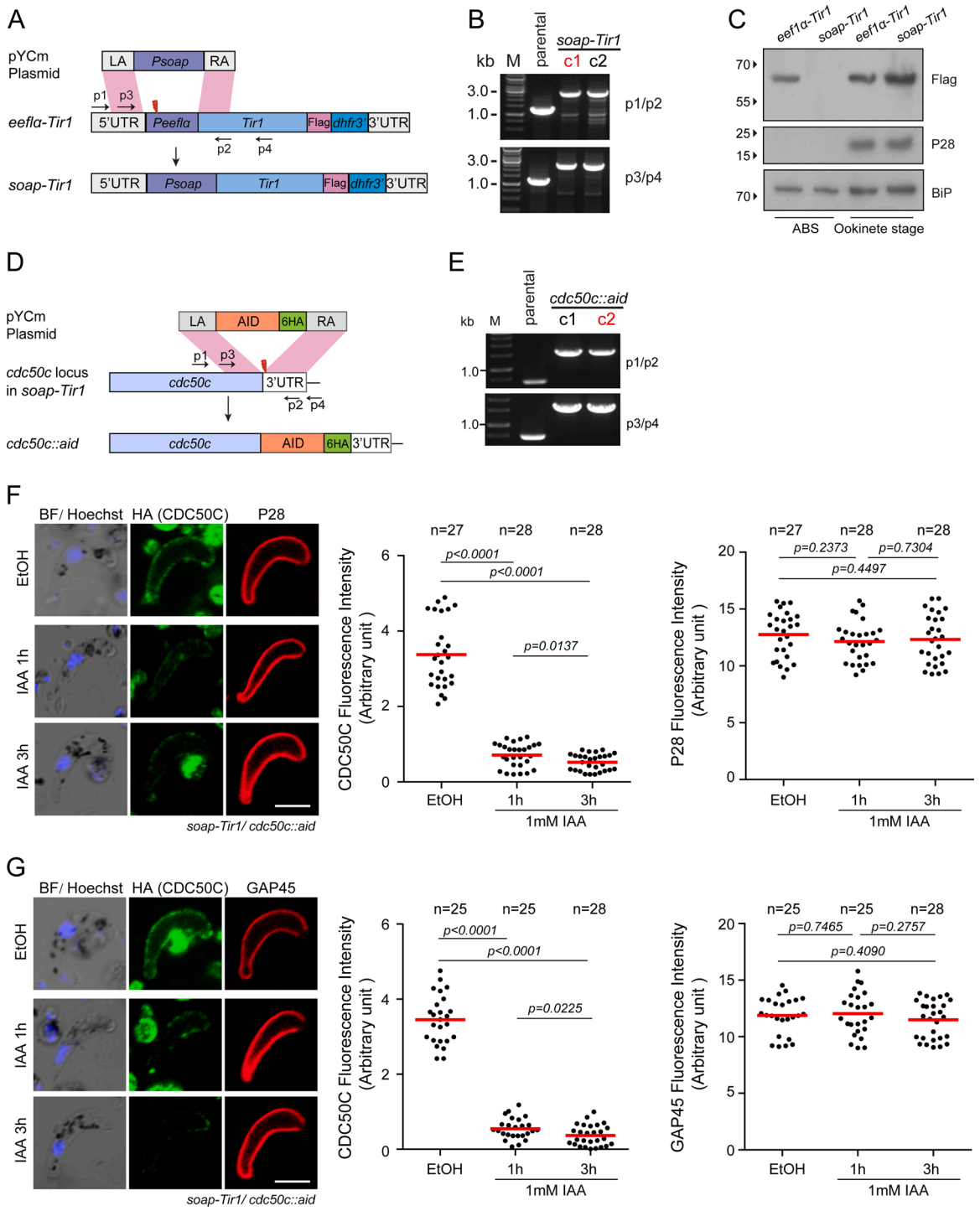

Fig. 3. Conditional depletion of the CDC50C protein in the *soap-Tir1* line stably expressing the *Tir1* under the promoters of *soap*.

A. Diagram of CRISPR/cas9 mediated replacement of the *eef1a* promoter with the *soap* promoter in the *eef1a-Tir1* line, resulting the *soap-Tir1* line. "p" indicates PCR primers. Red thunderbolt indicates sgRNA recognition site.

B. Genotyping of the parasite *soap-Tir1*. Two parasite clones (c) were obtained, and the clone indicated with red letter is used for further analysis.

C. Western blot analysis indicates the expression of transgenic Flag-tagged Tir1 protein in the ookinetes, but not the schizonts. BiP and P28 (ookinete specific protein) serve as the controls.

D. Diagram of CRISPR/cas9 mediated tagging of the *cdc50c* gene with the AID::6HA motif in the *soap-Tir1* line. "p" indicates PCR primers.

E. Genotyping of the strain *soap-Tir1/cdc50c::aid*. Two parasite clones (c) were obtained, and the clone indicated with red letter is used for further analysis.

F-G. Efficient depletion of CDC50C-AID in the *soap-Tir1/cdc50c::aid* ookinetes treated with 1 mM auxin, as measured by IFA detecting both CDC50C and P28 proteins (F) or CDC50C and GAP45 proteins (G). EtOH treatment serves as the control. Right panels are the quantification of fluorescent signal intensity of proteins. Red lines show the mean value. n is the number of cells analyzed in each group. Two-tailed unpaired Student's *t*-test.

abundance of AID::6HA peptide reappeared by 3 h, which could be efficiently inhibited by increasing auxin concentration to 4 mM (Fig. S2C).

Next, we explored if the *eef1a-Tir1* line is suitable for AID-based degradation of the endogenous protein. We selected *P. yoelii* gene *cdc50c* (PY17X_0514500) (Fig. 2A), which encodes a putative membrane protein with two transmembrane segments and a large exocytosolic loop. CDC50C is expressed at several development stages, including asexual blood stage, gametocyte, and ookinete of the *P. yoelii* [24]. To tag the *cdc50c* with the *aid* motif by the CRISPR/Cas9 methods, we constructed a pYCm plasmid containing the sequence encoding AID::6HA degron flanked by 518 bp of the 3′ coding region and 529 bp of the 3′UTR of the *cdc50c* gene as left and right homologous templates, respectively (Fig. 2B). After transfection of the *eef1a-Tir1* strain, drug selection, and parasite cloning, two single clones (*eef1a-Tir1/cdc50c::aid*) were obtained. Correct integration of homologous templates into the *cdc50c* locus were confirmed by PCR (Fig. 2C). In the purified schizonts and gametocytes from the *eef1a-Tir1/cdc50c::aid* parasites, immunoblot analyses detected a major band at ~94 kD that matches the expected size of the CDC50C::AID::6HA (50C-AID) fusing protein (Fig. 2D). We then asked if the endogenous CDC50C fused with the AID::6HA degron (50C-AID) could be depleted in an auxin-dependent manner. Immunoblot analyses showed that 1 mM auxin induced efficient depletion of 50C-AID, but not the BiP and enolase within 30 min of treatment in the parasites at the asexual blood stage (Fig. 2E), schizonts (Fig. 2F), and gametocytes (Fig. 2G). We also chose to target another *Plasmodium* protein Fbxo1 (PY17X_1120000), whose function is currently unknown. Taking a similar CRISPR/Cas9 approach, we obtained the *eef1a-Tir1/fbxo1::aid* parasite clone (Fig. S3A–C). Immunoblot and immunofluorescence analysis (IFA) showed that auxin treatment triggered rapid depletion of FBXO1 in both the schizonts and gametocytes (Fig. S3D–F). These results validate the *P. yoelii eef1a-Tir1* line for degrading endogenous proteins in the parasites of asexual blood stage and gametocytes.

We noticed that in the ookinetes of *eef1a-Tir1/cdc50c::aid* parasites, we failed to detect a clear depletion of 50C-AID even after 3 h of auxin treatment (Fig. S4), which is likely due to the relatively lower Tir1 expression in the ookinetes compared to that in the asexual blood stage and gametocytes (Fig. 1C). Therefore, we attempted to engineer a transgenic line to achieve higher Tir1 expression in the ookinetes. To that end, we used the promoter of *soap* (PY17X_1040200), a gene specifically and highly expressed in the ookinetes and early oocysts [26], to drive the *Tir1* expression. Using the CRISPR/Cas9 method, we replaced the *eef1a* promoter with the *soap* promoter (1999bp) at the upstream of *Tir1* in the *eef1a-Tir1* parasites (Fig. 3A), and generated a new line designated as *soap-Tir1* (Fig. 3B). As expected, the Tir1 protein is highly expressed in the ookinetes, but not in the asexual blood stage of the *soap-Tir1* parasites (Fig. 3C). We then tagged the endogenous CDC50C with the AID::6HA degron in the *soap-Tir1* parasite to test the auxin-based degradation of CDC50C (Fig. 3D and E). Because it is difficult to obtain the ookinetes with high purity for immunoblot analysis, we performed IFA to assess the auxin-induced protein degradation in the individual ookinete. Notably, auxin treatment (1 mM for 1 h) induced drastic depletion of CDC50C, but not P28 (parasite plasma membrane protein) and GAP45 (parasite inner membrane complex protein) in the ookinetes (Fig. 3F and G). Importantly, the CDC50C protein level in the parasites at the asexual blood stage seemed unaffected with auxin incubation at the same culture (Fig. 3F and G), which is consistent with no appreciable expression of the Tir1 at this stage (Fig. 3C). Together, these results consolidate the AID system for degrading endogenous proteins in the *P. yoelii*.

The plant derived auxin-AID system is a robust chemical-genetic based method for conditionally manipulating the protein level *via* proteasome-mediated degradation [15,16]. However, this system has not been adapted in the malaria parasites other than the *P. berghei* [18]. Our initial design of this study was to generate transgenic *P. yoelii* lines expressing exogenous Tir1 driven by two different constitutive promoters (*hsp70* and *eef1a*) respectively. We readily obtained the *eef1a--Tir1* line but not the *hsp70-Tir1*, which is unexpected considering successful generation of similar transgenic lines of the *P. berghei* [18]. Overexpressing Tir1 with multiple episomal plasmid copies likely render the parasites at the asexual blood stage more susceptible to the selection pressure imposed by drug treatment, an essential procedure of CRISPR/Cas9 screening [2]. In addition to the *eef1a-Tir1* line, we generated another marker-free transgenic *P. yoelii* line *soap-Tir1*, which stably expresses Tir1 under the promoter of a developmental stage-specific gene *soap* [26]. These two lines (*eef1a-Tir1* and *soap-Tir1*) develop normally during the parasite life cycle. In these transgenic lines, we used the CRISPR/Cas9 method to tag *cdc50c* and *fbxo1* with the AID motif and fulfilled efficient protein degradation upon auxin treatment. The *eef1a-Tir1* line allows efficient degradation of AID-tagged endogenous protein in the parasites at the asexual blood stages and gametocytes, while the *soap-Tir1* line is capable of doing so in the ookinetes. These lines will be valuable tools for studying the *P. yoelii*-based parasite biology. Noteworthy, the *Oryza sativa* TIR1 (OsTIR1) is the most commonly used auxin receptor F-box protein in combination with degrons deriving from *Arabidopsis thaliana* IAA17 (AtIAA17) [16–19]. However, the current AID systems can severely degrade target proteins before auxin addition and suffer from inefficient auxin-inducible depletion in a target-specific manner [16,27,28]. These pitfalls substantially limited the AID applications. Some significant improvements of AID system has been made with both minimal basal degradation without auxin and rapid auxin-inducible depletion of target proteins in the human cell lines [29,30]. Adaptation of these improvements for better AID application in the *Plasmodium* parasites await for further investigation.

CRediT authorship contribution statement

Chuanyuan Liu: Methodology, Validation, Investigation. **Zhenke Yang:** Methodology, Validation, Investigation. **Mengya Cai:** Methodology. **Yang Shi:** Methodology. **Huiting Cui:** Supervision. **Jing Yuan:** Supervision, Funding acquisition, Writing - review & editing.

Declaration of Competing Interest

The authors report no declarations of interest.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (31772443, 31872214, 31970387), the Natural Science Foundation of Fujian Province (2019J05010), and the 111 Project sponsored by the State Bureau of Foreign Experts and Ministry of Education of China (BP2018017).

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.2020.111346.

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