Systematic CRISPR-Cas9-Mediated Modifications of *Plasmodium yoelii* ApiAP2 Genes Reveal Functional Insights into Parasite Development

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ABSTRACT  Malaria parasites have a complex life cycle with multiple developmental stages in mosquito and vertebrate hosts, and different developmental stages express unique sets of genes. Unexpectedly, many transcription factors (TFs) commonly found in eukaryotic organisms are absent in malaria parasites; instead, a family of genes encoding proteins similar to the plant *Apetala2* (ApiAP2) transcription factors is expanded in the parasites. Several malaria ApiAP2 genes have been shown to play a critical role in parasite development; however, the functions of the majority of the ApiAP2 genes remain to be elucidated. In particular, no study on the *Plasmodium yoelii* ApiAP2 (PyApiAP2) gene family has been reported so far. This study systematically investigated the functional roles of PyApiAP2 genes in parasite development. Twenty-four of the 26 PyApiAP2 genes were selected for disruption, and 12 were successfully knocked out using the clustered regularly interspaced short palindromic repeat–CRISPR-associated protein 9 (CRISPR-Cas9) method. The effects of gene knockout (KO) on parasite development in mouse and mosquito stages were evaluated. Ten of 12 successfully disrupted genes, including two genes that have not been functionally characterized in any *Plasmodium* species previously, were shown to be critical for *P. yoelii* development of sexual and mosquito stages. Additionally, seven of the genes were labeled for protein expression analysis, revealing important information supporting their functions. This study represents the first systematic functional characterization of the *P. yoelii* ApiAP2 gene family and discovers important insights on the roles of the ApiAP2 genes in parasite development.

IMPORTANCE  Malaria is a parasitic disease that infects hundreds of millions of people, leading to an estimated 0.35 million deaths in 2015. A better understanding of the mechanism of gene expression regulation during parasite development may provide important clues for disease control and prevention. In this study, systematic gene disruption experiments were performed to study the functional roles of members of the *Plasmodium yoelii* ApiAP2 (PyApiAP2) gene family in parasite development. Genes that are critical for the development of male and female gametocytes, oocysts, and sporozoites were characterized. The protein expression profiles for seven of the PyApiAP2 gene products were also analyzed, revealing important information on their functions. This study provides expression and functional information for many PyApiAP2 genes, which can be explored for disease management.

KEYWORDS  genetic modification, malaria, mice, mosquito, transcription
Plasmodium parasites are single-cell organisms that have a complex life cycle involving mosquitoes and vertebrate hosts (1). Once sporozoites from an infected mosquito are injected into the dermis of a vertebrate host, the parasites quickly enter the liver and develop within hepatocytes. After schizogonous development in the liver, merozoites released from hepatocytes invade erythrocytes, starting the intraerythrocytic schizogony that produces more merozoites. To be transmitted by a mosquito, the parasites can then switch to sexual development, forming male and female gametocytes. When a second mosquito takes blood from an infected patient, male and female gametocytes differentiate into male and female gametes that fertilize to produce zygotes and oocysts. The motile oocysts penetrate the midgut wall to develop into oocysts containing thousands of sporozoites in approximately 12 to 15 days. Mature sporozoites then invade salivary glands (SGs) to infect a new vertebrate host when the mosquito bites again. The development and transformation of different developmental stages of malaria parasites are tightly controlled by gene expression regulation (2, 3). Although good progress in studying malaria parasite gene expression regulation has been made in recent years, large gaps in our knowledge of the mechanisms of parasite development and gene expression regulation are still present.

Previous analyses of apicomplexan genomes have revealed an unexpected paucity of transcription factors (TFs) commonly found in other eukaryotes; however, a lineage-specific expansion of a family of proteins similar to Apetala2 (ApiAP2) proteins found in many plants (4–6) was discovered in these apicomplexan organisms (7). There are 26 ApiAP2-related genes in the mouse malaria parasites, such as Plasmodium berghei and Plasmodium yoelii, and 27 genes in the human parasite Plasmodium falciparum (8, 9). Most of the malaria ApiAP2 family members have 1 to 3 DNA binding domains of approximately 60 amino acids (6, 10). The malaria ApiAP2 gene families have received considerable attention in recent years, and studies have shown critical roles of some ApiAP2 genes in parasite development. In a comprehensive survey, DNA binding specificities for the P. falciparum AP2 proteins were identified (11). Additionally, several members of the family were shown to play important roles in the growth and development of sexual or mosquito stages. For example, AP2-G and AP2-G2 have been shown to be critical for the initiation and progression of gametocytogenesis (12–14), although differences in parasite development and the gene functions exist between species of Plasmodium parasites. P. berghei AP2-O (PbAP2-O) was found to be essential for oocyst development and could activate the expression of ~500 genes involved in morphogenesis, locomotion, midgut penetration, protection against mosquito immunity, and preparation for subsequent oocyst development (15, 16). An ApiAP2 gene expressed in late oocysts and SG sporozoites was shown to be a major transcription factor that regulates gene expression in P. berghei’s sporozoite stage (17). Another P. berghei ApiAP2 gene, designated ap2-l, was found to play a critical role in the liver stage development of the parasite. Parasites without ap2-l proliferated normally in blood and in mosquitoes; however, the ability of the parasite to infect liver cells was greatly reduced (18). Interestingly, another member of the P. falciparum ApiAP2 family, P. falciparum SIP2 (PfSIP2), was found to interact specifically with cis-acting SPE2 motif arrays in subtelomeric domains, suggesting the involvement of an ApiAP2 protein in heterochromatin formation and genome integrity (19). Recently, a P. falciparum parasite-specific transcription factor, PfAP2-l, was found to be responsible for regulating the expression of genes involved in red blood cell (RBC) invasion (20). A noncanonical member of the ApiAP2 family of transcription factors, PfAP2Tel, was identified as a component of the P. falciparum telomere-binding protein complex, possibly playing a role in the expression of telomeric genes (21). Finally, in a systematic knockout (KO) screen in P. berghei, Modrzynska et al. were able to disrupt 11 ApiAP2 genes and identified 10 genes that were important for mosquito transmission, including four that were critical for the formation of infectious oocysts and three required for sporogony (9). There were also many P. berghei ApiAP2 genes that resisted at least four disruption attempts with up to two different vector designs and were considered essential for parasite survival and/or proliferation of blood stage parasites (9).
In this study, we attempted to disrupt 24 of the 26 ApiAP2 genes in the mouse malaria parasite *P. yoelii* using the clustered regularly interspaced short palindromic repeat–CRISPR-associated protein 9 (CRISPR-Cas9)-based methods we described previously (22), and we were able to knock out 12 of these 24 genes, including 3 genes homologous to genes that were not disrupted in *P. berghei* (9). We infected mice and mosquitoes with the mutant parasites to characterize phenotypic changes in parasite development, leading to functional characterization of two new *P. yoelii* ApiAP2 (PyApiAP2) genes that play important roles in gametocytogenesis and ookinete development, respectively. We also successfully tagged seven PyApiAP2 proteins with sextuple hemagglutinin (6×HA) tags or the red fluorescent protein mCherry and revealed previously unknown protein expression profiles relevant to their functions in parasite development. These results provide new insights into ApiAP2 gene functions and their roles in *P. yoelii* development in mosquito and vertebrate hosts.

**RESULTS**

**Systematic disruption and tagging of PyApiAP2 genes.** To investigate the functions of the PyApiAP2 gene family in parasite development, we attempted to disrupt 24 of the 26 PyApiAP2 genes in the parasite genome, excluding the orthologs of *Pbap2-sp* and *Pbap2-l*, whose functions were described when we initiated the project (17, 18). We were able to knock out 12 of the 24 genes (see Fig. S1A to Q and Table S1 in the supplemental material), including three PyApiAP2 genes (PY17X_1317000, PY17X_1417400, and PY17X_0523100) whose orthologs in *P. berghei* were either resistant to disruption or not attempted (9). We carefully evaluated the morphologies of asexual/sexual stages in ICR mice and sexual stages in *Anopheles stephensi* mosquitoes for the 12 gene KO mutants. We also successfully tagged six PyApiAP2 proteins (PyAP2-G, PyAP2-G3, PyAP2-O2, PyAP2-O3, PyAP2-O4, and PyAP2-O5) with 6×HA tags and PyAP2-O with mCherry to investigate protein expression and localization in the parasites (Fig. S1R to AA). There were 12 PyApiAP2 genes (PY17X_0104500, PY17X_1231600, PY17X_1209100, PY17X_0941600, PY17X_0911000, PY17X_1361700, PY17X_1456200, PY17X_0111100, PY17X_0113700, PY17X_0838600, PY17X_0934300, and PY17X_1405400) that could not be disrupted even after 4 to 12 independent transfections and selections (Table S1). The orthologs of these 12 genes in *P. berghei* also resisted disruption attempts (9) and are likely to be essential for parasite viability or affect the growth of these rodent parasites in the mouse. Further investigations are necessary to dissect the functional roles of these genes, including tagging the genes for protein expression analysis. Transcriptome analysis of *P. berghei* showed constitutive expression of these genes in the blood stages, suggesting that they play important roles in parasite development (23).

**Functional characterization of three PyApiAP2 genes essential for gametocyte development.** Two ApiAP2 genes (*Pfap2-g/Pbap2-g* and *Pbap2-g2*) have been shown to play a critical role in gametocytogenesis in *P. falciparum* and/or *P. berghei* (9, 12–14). To investigate whether the genes are also critical to *P. yoelii* gametocytogenesis, we used the CRISPR-Cas9 method to disrupt the *Pyap2-g* and *Pyap2-g2* genes. We employed two strategies to disrupt the *Pyap2-g* genes. One strategy was to delete a segment of the 5′ coding region of the *Pyap2-g* gene, generating a Δ*Pyap2-g* parasite. This procedure inserted a stop codon and generated a frameshift for the remaining coding region, which was confirmed after DNA sequencing of the cloned parasites. The other strategy was to replace the whole coding region with a gene encoding green fluorescent protein (GFP), generating a *Pyap2-gΔ/gfp* parasite (Fig. S2A and B). Both *Pyap2-g* KO parasites produced no gametocytes (Fig. S2C) or oocysts (Fig. S2D). We also tagged the PyAP2-G protein with 6×HA and detected PyAP2-G protein in the nucleus of some ring- and trophozoite-like parasites via immunofluorescence assay (IFA), suggesting committed young gametocytes (Fig. S2E). We counted the numbers of ring- and trophozoite-like parasites via immunofluorescence assay (IFA), suggesting committed young gametocytes (Fig. S2E). We counted the numbers of ring- and trophozoite-like parasites with nuclear PyAP2-G expression and showed that approximately 3.5% (93/2,667) of ring- or early trophozoite-like but no late trophozoites/schizonts (0/2,760) were positive for nuclear PyAP2-G expression. This percentage of PyAP2-G-expressing
The ortholog of this gene is resistant to genetic disruption in gametocyte development. Plasmodium P. yoelii development in role in gametocyte development, and we therefore call the gene Pyap2-g3 parasite clone that was engineered to express mCherry-tagged sexual-specific protein with the sequence encoding 6HA and observed protein expression using IFA and Western blotting (Fig. S3B). The strong cytoplasmic expression of PyAP2-G3 in asexual stages also suggests that this protein may recognize cytoplasmic signals and relay the signals into the nucleus, where they interact with PyAP2-G and turn on the process of gametocytogenesis; this idea requires further investigation.

Functional characterization of three PyApiAP2 genes affecting ookinete development. Another interesting PyApiAP2 gene we discovered is PY17X_1317000, which contributes to ookinete motility and is essential for oocyst development, a finding that has not been described previously. Parasites without this gene had normal male and female gametocytes (Fig. 1A), day 8 oocysts (Fig. 1B), and SG sporozoites (Fig. 1C), although the absence of Pyap2-g3 did not affect asexual growth (Fig. 1D). We also evaluated the effects of Pyap2-g KO on Pyap2-g3 expression and vice versa. Disruption of Pyap2-g did not significantly affect Pyap2-g3 expression; however, Pyap2-g3 KO significantly reduced Pyap2-g expression (Fig. 1E and F). We next tagged PyAP2-G3 with 6×HA at the N-terminal end and detected protein expression using IFA and Western blotting (Fig. 1G and H). Tagging the protein with 6×HA did not significantly affect the development of male and female gametocytes (Fig. 3A), ookinete conversion (Fig. 3B), the numbers of oocysts (Fig. 3C) or SG sporozoites (Fig. 3D). Interestingly, PyAP2-G3 is strongly expressed in the cytoplasm and to a lesser degree in the nuclei of asexual stages and gametocytes (all the blood stages), but not in ookinetes (Fig. 1G; Fig. 3E). The strong cytoplasmic expression of PyAP2-G3 in asexual stages also suggests that this protein may recognize cytoplasmic signals and relay the signals into the nucleus, where they interact with PyAP2-G and turn on the process of gametocytogenesis; this idea requires further investigation.
oocyst development (Fig. S4A and B). Seventy-two hours after feeding mosquitoes, the mCherry signals were greatly reduced in the Pyap2-o5 KO parasites, suggesting dying parasites (Fig. S4C). The results are consistent with the observations of reduced ookinete motility in vitro and the lack of mature oocysts in the mosquito midgut. Taken together, these results clearly demonstrate that Pyap2-o5 plays a role in ookinete motility and early oocyst development.

A second PyApiAP2 gene that can affect ookinete development is PY17X_1017000, which has been designated ap2-o3 (9). Although disruption of this gene did not significantly affect male or female gametocyte development (Fig. 3A) or exflagellation (Fig. 3B), no ookinetes with normal morphology (Fig. 3C) and no normal ookinetes (Fig. 3D) or SG sporozoites (Fig. 3E) were observed in the Pyap2-o3 KO parasite. We also tagged the gene with the sequence encoding 6×HA and performed IFA to investigate protein expression in various developmental stages. The protein was expressed in the nuclei of gametocytes, zygotes, and oocysts but not in asexual stages and ookinetes.
Costaining of gametocytes with anti-HA antibody (PyAP2-O3) and anti-\(\alpha\)-tubulin antibody (a male gametocyte-specific marker) showed that PyAP2-O3 was only expressed in female gametocytes (Fig. 3G).

We also disrupted Pyap2-o and showed normal gametocyte development, but no normal ookinetes were observed (Fig. S5A and B), confirming an essential role of Pyap2-o in \(P. yoelii\) ookinete development. Tagging PyAP2-O with mCherry showed protein expression in zygotes and ookinetes but not in asexual stages, gametocytes, or oocysts (Fig. S5C).

The protein expression patterns of the three genes affecting ookinete development (Pyap2-o, Pyap2-o3, and Pyap2-o5) are intriguing. Whereas PyAP2-O was only expressed in zygotes and ookinetes, PyAP2-O3 and PyAP2-O5 were not detectable at the ookinete (Fig. 3F).
**FIG 3** Phenotypic and expression analyses of Pyap2-o3 in blood and mosquito stages. (A and B) Male and female gametocytemia (A) and exflagellation rate (B) from blood samples on day 3 postinfection of WT or Pyap2-o3 KO parasites. (C) Ookinete conversion rate (normal, mature ookinete; abnormal, retort ookinete) counted on Giemsa-stained blood films from 24-h *in vitro* cultures. *P* values were determined using the Mann-Whitney test; **, *P* < 0.01. The ookinete conversion rate was obtained by dividing the numbers of retort and mature ookinetes by the number of female gametocytes among the same number of RBCs. (D) Numbers of day 4 or day 8 oocysts per mosquito from WT- or Pyap2-o3 KO-infected mosquitoes. (E) Numbers of day 14 salivary gland (SG) sporozoites per infected mosquito. Mean values and SEM were calculated from the results of three independent experiments or counts of oocysts or sporozoites from 20 to 30 mosquitoes. ND, not detected. (F) Immunofluorescence assay (IFA) of PyAP2-O3 protein expression in various developmental stages detected using anti-HA antibody. Scale bar = 5 μM. (G) IFA images of male and female gametocytes double stained with anti-HA and anti-α-tubulin antibodies. Scale bar = 5 μM. The data are representative of three independent experiments or three mice.
stage (Fig. 2H and 3F; Fig. S5C). Pbap2-o3 KO parasites were shown to have gene clusters highly enriched for genes known to be transcribed in female gametocytes (macrogametocytes) but not translated until gametocyte activation after entering the mosquito (9, 27). One example is cluster 31, which includes genes encoding P25 and P28 (9, 27). In the ookinete cultures of the Pbap2-o3 KO parasite, these genes were not downregulated, suggesting a failure to repress transcription or reduce transcript turnover (9). These observations led us to examine P28 protein expression in Pyap2-o3 KO parasites. Our data showed significantly (~72%) reduced numbers of cells expressing mCherry-labeled P28, reduced signal intensity in P28-expressing cells (~69%), and lower protein levels (~55% reduction after quantification of protein bands on Western blots) in activated Pyap2-o3 KO gametocytes (Fig. S5D to F). The smaller-scale reduction in protein signal could be due to the different methods (fluorescent versus colorimetric) and antibodies used. These results support the idea that Pyap2-o3 may regulate (activate) the transcription of genes that release the repression of translation of P28 and the other genes important for ookinete development.

Functional characterization of two PyApiAP2 genes affecting oocyst development. We also evaluated two PyApiAP2 genes whose orthologs have been shown to be critical for ookinete development in P. berghei (Pbap2-o2 and Pbap2-o4). Parasites without Pbap2-o2 were shown to have greatly reduced ookinete numbers, and those without Pbap2-o4 had normal ookinetes but few oocysts (9). Disruption of Pyap2-o2 did not significantly affect the numbers of male and female gametocytes, exflagellation, or the ookinete conversion rate (Fig. 4A to C), but the numbers of day 8 oocysts, day 12 sporozoites in oocysts, and day 14 SG sporozoites were dramatically reduced in the Pyap2-o2 KO parasites (Fig. 4D to F). We also disrupted this gene in the parasite with mCherry-tagged P28 and obtained similar results (Fig. S6A to D). However, the numbers of parasites in the mosquito midgut from day 1 to day 3 after feeding were similar between wild-type (WT) and Pyap2-o2 KO parasites (Fig. S6E), suggesting normal early oocyst development. Infection of mice with SG sporozoites of the Pyap2-o2 KO parasite could still lead to blood stages (Table S2A). Our results are slightly different from those for Pbap2-o2; Pyap2-o2 KO does not affect ookinete conversion or development. However, the effect of reducing oocyst and sporozoite numbers are the same in both P. yoelii and P. berghei. We also tagged PyAP2-O2 with 6×HA and analyzed the protein expression using IFA. PyAP2-O2 protein was also expressed in the nuclei of asexual stages, gametocytes, zygotes, retort ookinetes, and oocysts but not in ookinetes (Fig. 4G). The results show that Pyap2-o2 has minimal roles in the development of asexual stages, gametocytes, and ookinetes but is critical for oocyst and sporozoite development.

We next investigated the roles of Pyap2-o4 (PY17X_1369400) in oocyst and sporozoite development. As reported in P. berghei previously (9), disruption of this gene did not significantly affect the numbers of male and female gametocytes, exflagellation, or ookinete motility and morphology (Fig. 5A to E), but it completely blocked oocyst and sporozoite development (Fig. 5F and G). Additionally, we tagged the PyAP2-O4 protein with 6×HA and showed that the protein was expressed in the nuclei of all sexual and mosquito stages (some cytoplasmic expression in gametocytes and oocyst) but not in blood stages (Fig. 5H).

We also disrupted Pyap2-o4 in the parasite with mCherry-tagged P28 to investigate the effects of Pyap2-o4 KO on early oocyst development. We showed that gametocytes developed normally, but no day 8 oocysts were found (Fig. S7A and B). Parasite development in mosquitoes in the first 24 h appeared to be normal, but the parasites started to die after 48 h, with decreasing numbers and sizes of mCherry-expressing parasites observed (Fig. S7C to E). These results show that Pyap2-o4 is essential for early oocyst development (within 48 h after entering mosquitoes), which is consistent with the observations in P. berghei (9).

Functional characterization of two PyApiAP2 genes essential for sporozoite development. We next investigated two PyApiAP2 genes whose orthologs in P. berghei have been shown to affect sporozoite development (9). Disruption of Pyap2-sp2
FIG 4 Characterization of Pyap2-o2 function and expression in blood and mosquito stages. (A to C) Male and female gametocytemia on day 3 postinfection (A), exflagellation rate (B), and ookinete conversion rate (C) from 24-h in vitro ookinete cultures of WT or Pyap2-o2 KO parasites. (D) Numbers of day 8 oocysts per mosquito. (E and F) Numbers of day 12 oocyst sporozoites (E) or day 14 salivary gland (SG) sporozoites (F). (Continued on next page)
or Pyap2-sp3 (PY17X_1123200) did not affect gametocyte development (Fig. 6A). The KO parasites also had approximately the same number of day 8 oocysts per mosquito (Fig. 6B), with day 8 and day 12 oocyst sizes similar to those of WT 17XNL parasites (Fig. 6C). However, no sporozoites were observed in the day 12 oocysts in mosquitoes infected with Pyap2-sp2 KO parasites; in contrast, the numbers of sporozoites in day 12 oocysts of Pyap2-sp3 KO parasites were not significantly reduced compared with the results for WT parasites (Fig. 6D). Neither Pyap2-sp2 nor Pyap2-sp3 KO parasites produced SG sporozoites (Fig. 6E). Whereas the oocysts of the Pyap2-sp2 KO parasite became vacuolated without any sporozoites (Fig. 6F), the day 12 sporozoites (F) per infected mosquito. Mean values and SEM were calculated from the results for three independent experiments or counts of oocysts or sporozoites from 20 to 30 mosquitoes. ***P < 0.001. (G) Immunofluorescence assay (IFA) of PyAP2-O4 protein expression in various developmental stages detected using anti-HA antibody. The data are representative of three independent experiments. ns, not significant.

(PY17X_1003200) or Pyap2-sp3 (PY17X_1123200) did not affect gametocyte development (Fig. 6A). The KO parasites also had approximately the same number of day 8 oocysts per mosquito (Fig. 6B), with day 8 and day 12 oocyst sizes similar to those of WT 17XNL parasites (Fig. 6C). However, no sporozoites were observed in the day 12 oocysts in mosquitoes infected with Pyap2-sp2 KO parasites; in contrast, the numbers of sporozoites in day 12 oocysts of Pyap2-sp3 KO parasites were not significantly reduced compared with the results for WT parasites (Fig. 6D). Neither Pyap2-sp2 nor Pyap2-sp3 KO parasites produced SG sporozoites (Fig. 6E). Whereas the oocysts of the Pyap2-sp2 KO parasite became vacuolated without any sporozoites (Fig. 6F), the day 12
oocysts of the Pyap2-sp3 KO parasites contained sporozoites with a morphology similar to that of WT parasites (Fig. 6G). Repeated infections of mice (3 ×) with the oocyst sporozoites from the Pyap2-sp3 KO parasite failed to establish infection (Table S2B), suggesting immature or defective oocyst sporozoites. These results suggest that Pyap2-sp2 is essential for early sporozoite development within oocysts, whereas Pyap2-sp3 is essential for sporozoite maturation later.

**Two nonessential PyApiAP2 genes.** There were two PyApiAP2 genes (PY17X_0523100 and PY17X_1323500) that were disrupted successfully, but the parasites without the genes showed no detectable developmental defects or phenotypes in either asexual or sexual stages. The ortholog of PY17X_1323500 was disrupted successfully in *P. berghei*, but not that of PY17X_0523100. Parasites without the two genes showed similar numbers of gametocytes (Fig. S7F), day 7 oocysts per mosquito (Fig. S7G), and day 14 SG sporozoites (Fig. S7H) compared with the results for WT parasites, and infection of mice with sporozoites from these mutant parasites resulted in blood stage infections (Fig. S7I). These results suggest redundancy for the functions of the two PyApiAP2 genes or that these genes could be involved in the response to environmental challenges that do not occur under controlled laboratory conditions.

FIG 6 Functional and phenotypic characterization of Pyap2-sp2 and Pyap2-sp3 in blood and mosquito stages. (A and B) Male and female gametocytemia on day 3 postinfection (A) and numbers of oocysts per mosquito on day 8 postfeeding (B) of WT, Pyap2-sp2 KO, or Pyap2-sp3 KO parasites. (C) Diameters of day 8 and day 12 oocysts from midguts of mosquitoes infected with WT, Pyap2-sp2 KO, or Pyap2-sp3 KO parasites. (D and E) Numbers of day 12 oocyst sporozoites (D) and day 14 salivary gland (SG) sporozoites (E) per mosquito after infection with WT, Pyap2-sp2 KO, or Pyap2-sp3 KO parasites. Mean values and SEM were calculated from the results of three independent experiments or counts of oocysts or sporozoites from 20 to 30 mosquitoes. (F and G) Representative DIC or merged images from WT or Pyap2-sp2 KO (F) and from WT and Pyap2-sp3 KO (G) parasites. Blue, Hoechst stain. Scale bar = 20 μm.
DISCUSSION

Our study presents the first systematic functional evaluation of the PyApiAP2 gene family. Previous studies have individually investigated 11 Plasmodium ApiAP2 genes functioning in sexual, mosquito, and liver stages in other species (12–19). In particular, a recent study attempted to knock out all of the ApiAP2 genes and succeeded in disrupting 10 genes in P. berghei (9). In addition to confirming the known Plasmodium ApiAP2 genes functioning in mosquito stages, our current study characterizes three additional genes that have never been functionally evaluated previously (PY17X_1317000, PY17X_1417400, and PY17X_0523100). Among the genes affecting sexual and mosquito stages, we investigated two new genes that could affect gametocyte (Pyap2-g3, or PY17X_1417400) and ookinete (Pyap2-o5, or PY17X_1317000) development, bringing to a total of 10 the number of PyApiAP2 genes that play critical roles in the development of nonerythrocytic stages. Based on the results of our gene disruption and expression analyses, as well as those of previous studies in P. falciparum and P. berghei, we can assign the functional roles of certain PyApiAP2 genes to specific time points of the parasite life cycle. Among the 24 genes studied here, 10 contribute to the development of sexual and mosquito stages and 12 are essential for parasite viability (Fig. 7A). Of the 10 nonerythrocytic-stage-related genes we characterized, three play a role in gametocyte development (Pyap2-g, Pyap2-g2, and Pyap2-g3), 3 are critical for ookinete development or motility (Pyap2-o, Pyap2-o3, and Pyap2-o5), 2 affect oocyst development (Pyap2-o2 and Pyap2-o4), and 2 are essential for oocyst and sporozoite development (Pyap2-sp2 and Pyap2-sp3 [Pyap2-sp was not studied here]). Our detailed characterizations of different mosquito stages allow functional placement of these genes at precise developmental time points in the parasite life cycle (Fig. 7B) and improve our understanding of PyApiAP2 gene functions.

We also attempted to tag all 12 genes that affect the development of nonerythrocytic stages with sequences encoding 6×HA or mCherry to study their protein expression. We were able to detect expressed tagged proteins from seven genes (Pyap2-g, Pyap2-g3, Pyap2-o, Pyap2-o2, Pyap2-o3, Pyap2-o4, and Pyap2-o5) (Fig. 7C) but failed to obtain parasites expressing the other five proteins with the 6×HA tag. Except for Pyap2-G3, which is also strongly expressed in the cytoplasm, and some cytoplasmic expression of PyAP2-O4 in gametocytes and oocysts, all seven PyApiAP2 proteins are expressed in the nuclei of parasites at various developmental stages, consistent with their predicted functions as transcription factors (5–7, 28). The protein expression patterns of the three genes affecting ookinete development (Pyap2-o, Pyap2-o3, and Pyap2-o5) are very interesting. Whereas PyAP2-O3 and PyAP2-O5 are not expressed at the ookinete stage, PyAP2-O is highly expressed at the ookinete stage (Fig. 7C). The mutually exclusive expression patterns of PyAP2-O3/-5 and PyAP2-O at the ookinete stage suggest that PyAP2-O expression may shut down the expression of PyAP2-O3 and PyAP2-O5 or that the expression of PyAP2-O3/-5 turns on the expression of PyAP2-O. There are over 500 genes that play a role in various biological processes, including oocyst development, and have PbAP2-O binding motifs at the 5′ untranslated region (UTR) (15, 16). Interestingly, the 5′ UTR of Pbap2-o3 has a PbAP2-O binding motif, but the 5′ UTR of Pbap2-o5 does not (15, 16). These genes could control the expression of different sets of genes critical for ookinete and oocyst development. Previous studies show that many genes that are transcribed in macrogametocytes but not translated until gametocytes enter the mosquito are highly enriched in Pbap2-o3 KO ookinete culture (9, 27), leading to the suggestion that Pbap2-o3 may be required for zygotes to progress successfully beyond meiosis (9). The protein expression pattern of PyAP2-O3, in particular its expression in female gametocytes only, and reduced P28 protein expression in the Pyap2-o3 KO parasite are consistent with a hypothesis that PbAP2-O3 is expressed to regulate factors that are involved in translational repression of genes critical for female gamete and ookinete development. It would be interesting to dissect the roles of Pyap2-o, Pyap2-o3, and Pyap2-o5 in regulating each other’s expression and the expression of genes important for ookinete development.
It is not clear why PyAp2-o2 and PyAp2-o5 are expressed at asexual and/or early gametocyte stages, and disruptions of these two genes did not affect the development of asexual stages or gametocytes. Similarly, PyAp2-o2 and PyAp2-o4 were expressed in gametocytes, and again, gene disruption had no effect on gametocyte development.

One possible explanation for the lack of correlation in the protein expression of these ApiAP2 genes and their functional effects on parasite development is that the developmental defects observed after gene KO have their origins in gene expression regulation at the earlier stage, as suggested previously (9), and some downstream genes regulated by these transcription factors (TFs) are responsible for determining parasite development. Indeed, changes in the expression of many genes were observed in gametocytes after disruption of Pbap2-o2 and Pbap2-o3 (9), consistent with our observations of PyAP2-O2, PyAP2-O3, PyAP2-O4, and PyAP2-O5 protein expression in gametocytes. These results also suggest that some ookinete-specific genes, such as Pyap2-o, need to be dynamically regulated, with suppression at the gametocyte and zygote stages, expression at the ookinete stage, and then suppression again at the oocyst stage, which is regulated by other PyApiAP2 genes, possibly by Pyap2-o3 and Pyap2-o5.
Our results also demonstrate that PY17X_1417400 (Pyap2-g3) is a gene that plays an important role in gametocyte development. Interestingly, the insertion of a transposon into the coding sequence of the \textit{P. falciparum} ortholog gene (PF3D7_1317200) resulted in the absence of gametocytes, suggesting that it might play a role in gametocytogenesis; however, the role of the ortholog of this gene in gametocytogenesis could not be conclusively determined because the parasite line containing a transposon insertion in the PF3D7_1317200 gene also had a missense mutation in the Pfap2-g gene (24, 25). Our data clearly showed that disruption of Pyap2-g3 significantly reduced the number of gametocytes, leading to the absence of sporozoites even though a small number of day 8 oocysts were observed (Fig. 1). Importantly, we also showed that disruption of Pyap2-g3 could significantly reduce Pyap2-g transcription but not the other way around. Our observations are consistent with the report that disruption of Pfap2-g did not affect the expression of PF3D7_1317200 (ortholog of Pyap2-g3) (13). The strong expression of PyAP2-G3 in asexual stages is consistent with a hypothesis that PyAP2-G3 may function upstream from PyAP2-G and play a role in sensing cytoplasmic signals to activate pathways of gametocytogenesis. Indeed, many transcription factors, such as IRFs and STATs in type I interferon signaling, are able to shuttle between the cytosol and nucleus. Domain and motif searches of PyAP2-G3 reveal only an ApiAP2 domain located in the middle of the protein and an rpoC2 domain (RNA polymerase beta subunit) at the C-terminal end. No other domains with known binding specificities are present in PyAP2-G3, although we cannot exclude other unknown functional domains in this large protein (278 kDa). However, one of the characteristics of a parasite line that does not make gametocytes is that it generally grows more quickly than a line that does, and the Pyap2-g3 KO parasites grow similarly to the WT 17XNL line (Fig. 1D), suggesting that PyAP2-G3 may not play a role in sexual commitment or function downstream from PyAP2-G. Whether PyAP2-G3 is truly functioning upstream from PyAP2-G requires further investigation. Additionally, it would be interesting to investigate how these Pyap2-g/-g2/-g3 genes regulate each other in gametocytogenesis.

The observation that the schizonts of \textit{P. yoelii} and \textit{P. berghei} do not express ApiAP2-G, in contrast to the observation for the \textit{P. falciparum} ortholog (13), is interesting. This observation suggests some important differences in early sexual development between \textit{P. falciparum} and the rodent parasites \textit{P. yoelii} and \textit{P. berghei}, which requires further investigation. This observation is not surprising, considering the existing differences in the processes of sexual development between \textit{P. yoelii}/\textit{P. berghei} and \textit{P. falciparum}; it takes 15 days for \textit{P. falciparum} gametocytes to mature, whereas \textit{P. yoelii} gametocytes take 2 days.

Another interesting ApiAp2 gene we discovered is PY17X_1317000 (Pyap2-o5). The ortholog of this gene could not be disrupted in the \textit{P. berghei} study and was considered an essential gene for asexual-stage development (9). Using the CRISPR-Cas9 method, we were able to knock out the Pyap2-o5 gene in both WT 17XNL and mCherry-tagged 17XNL parasites and showed that disruption of this gene could significantly reduce ookinete motility and completely block oocyst and sporozoite development (Fig. 2; Fig. S4). Similar to Pyap2-o2, this gene is also expressed in asexual stages, including early schizonts.

Compared with the results of the ApiAP2 gene disruption screen of \textit{P. berghei}, we also successfully disrupted three additional genes. The differences in the gene KO results could be due to the differences in genes required for parasite development between parasite species; e.g., a gene could be essential in \textit{P. berghei} but not for \textit{P. yoelii}. One example of an ApiAP2 gene having slightly different functions between \textit{P. berghei} and \textit{P. yoelii} is ap2-o2. Parasites without Pbpap2-o2 had greatly reduced ookinete numbers (9), whereas Pyap2-o2-deficient and WT parasites had similar ookinete conversion rates. \textit{P. yoelii} and \textit{P. berghei} are closely related parasites, and approximately 90% of the predicted proteins in rodent malaria parasites (\textit{P. berghei}, \textit{P. yoelii}, and \textit{Plasmodium chabaudi}) have orthologs in primate malaria parasites (23, 29, 30), supporting evolutionarily conserved gene functions in \textit{Plasmodium} species. However, there are also large differences in gene families and in gene expression; for
example, whole-transcriptome shotgun sequencing (RNA-seq) analyses showed only approximately 65% correlation in blood stage gene expression between P. berghei and P. yoelii (23). Another possibility is the method used to disrupt the genes. In the P. berghei study, parasite DNA segments were cloned in a bacteriophage n15-based vector that can be modified efficiently using the lambda red method of recombineering, and genes were disrupted based on the traditional method of homologous crossover (9, 31). In our study, we used CRISPR-Cas9-mediated double-strand break and homologous repair (22). In theory, these two methods should yield comparable results because both methods have been used to delete genes efficiently. To sort out the reasons for the discrepancy, direct comparison of the two methods in knocking out a same set of genes in the same parasite may be necessary.

In addition to careful evaluation of parasite development in the mouse and mosquito models, we also used a parasite expressing mCherry-labeled P28 to visualize the early parasite stages before oocysts can be recognized in the mosquito midgut. We were able to visualize parasites from 24 h to 72 h after mosquito feeding and record the disappearance of some gene KO parasites. These experiments greatly improve the resolution of gene effects on parasite developmental stages. For example, we were able to show that the Pyap2-o4 KO parasites were smaller in size than WT parasites 48 to 72 h after mosquito feeding (Fig. S7).

There are two genes that can be disrupted in both P. berghei (9) and P. yoelii, but the resulting KO parasites showed no sign of a defect in any developmental stage (PY17X_0523100 affected oocyst and SG sporozoite numbers slightly but not significantly) (Fig. S7). In our hands, the parasites with one of the two genes disrupted could successfully complete their life cycles without any reduction in the numbers of gametocytes, oocysts, or SG sporozoites. The results suggest redundancy in the functions of these genes. Of great interest, the P. falciparum orthologues (PF14_0533 and PFD0985W) of these two proteins were shown to actually bind the same CACACA motif, although only one of the two DNA binding domains in the PY17X_0523100 ortholog (PFD0985W) binds the motif (11). These observations support functional redundancy for these two genes. In contrast, there are also 12 ApiAP2 genes that could not be disrupted in either P. berghei or P. yoelii, even after 4 to 12 independent attempts (our efforts) to disrupt the genes. Clearly, these genes may have a deleterious effect on asexual growth or are essential for asexual development, because the parasite has a haploid genome in blood stages. Further investigations are necessary to dissect the functions and the protein-protein interaction of AP2 proteins. Nonetheless, our study presents a comprehensive functional analysis of the P. yoelii ApiAP2 transcription factor family, providing important functional insights for the P. yoelii ApiAP2 genes and their roles in parasite development and gene expression.

MATERIALS AND METHODS

Ethics statement. All mouse experiments were performed in accordance with protocols (XMU-LAC20140004) approved by the Committee for the Care and Use of Laboratory Animals at the School of Life Sciences, Xiamen University.

Plasmid construction. To construct the vectors to disrupt the PyApiAP2 genes, we first amplified the 5’- and 3’-flanking genomic regions (400 to 700 bp) as left and right homologous arms using the primers listed in Table S3. The left and right arms were inserted into the restriction sites (HindIII/KpnI and NcoI for the left arm and XhoI and AflII/EcoRI for the right arm) in the pYC plasmid (22). Sequences for single guide RNAs (sgRNAs) were similarly annealed and ligated into the pYC plasmid.

To construct vectors for tagging PyApiAP2 genes with sequences coding for mCherry or 6×HA, we first amplified the C- or N-terminal part (300 to 800 bp) of the coding region as the left or right arm and 400 to 800 bp from the 5’ UTR or 3’ UTR following the translation stop codon as the right or left arm using the primers in Table S3. A DNA fragment encoding the mCherry or 6×HA tag was inserted between the left and right arms in frame with the gene of interest. For each gene, one sgRNA was designed to target the site close to the C- or N-terminal part of the coding region.

Malaria parasite strain and parasite transfection. All transfections were performed on the P. yoelii 17XNL strain. The parasites were propagated in ICR mice (female, 5 to 6 weeks old) purchased from the Animal Care Center, Xiamen University. The procedure for parasite transfection, pyrimethamine selection, and cloning were as described previously (22).

Exflagellation induction. Mice treated once with phenylhydrazine (3.5 μl/g or 25 mg/ml) were used to obtain blood with high gametocytemia. Male and female gametocytes were counted from Giemsa-
stained thin blood smears on day 3 postinfection. Gametocytemia was defined as the ratio of male or female gametocytes in infected RBCs (expressed as percentages). Three days postinfection (p.i.), the exflagellation rate was quantified as previously described after adding 2.5 μl of mouse tail blood to 100 μl of exflagellation medium (RPMI 1640 supplemented with 20% fetal calf serum [FCS] and 100 μM xanthurenic acid) containing 1 μl of 200 units/ml heparin. After 10 min of incubation at 22°C, the numbers of exflagellating microgametocytes (exflagellation centers) were counted using a hemocytometer, and the numbers of RBCs were estimated under a microscope. The percentage of RBCs containing microgametocytes (male gametocytemia) was calculated from Giemsa-stained smears. The number of exflagellation centers per 100 microgametocytes, or “exflagellation rate,” was then calculated. Results were obtained from three independent experiments.

**Ookinete culture in vitro and ookinete motility.** Ookinete culture and evaluation of ookinete motility were done according to the procedures described previously and in Text S1 in the supplemental material.

**Mosquito infection and observation of parasites in mosquitoes.** For mosquito infection, 50 female A. stephensi mosquitoes were allowed to feed on anesthetized infected mice that carried comparable numbers of gametocytes as determined by Giemsa staining for 20 min. Mosquito midguts were dissected on days 1, 2, and 3 p.i. Midguts were washed twice with phosphate-buffered saline (PBS) and then fixed with 4% paraformaldehyde–0.0075% glutaraldehyde on slides for 30 min. After three washes in PBS, cells were stained with Hoechst 33342. Ookinetes expressing mCherry, Hoechst 33342-labeled nuclei, and midgut epithelium cells were observed using a Zeiss LSM 780 laser-scanning confocal microscope.

**Counting oocysts and sporozoites.** Twenty mosquitos were dissected on days 4, 8, and 12 p.i., and oocysts in the midguts were counted under a microscope. To isolate day 12 oocysts, mosquito midguts were pulverized using a grinding rod to release midgut sporozoites. Salivary glands were isolated from 20 to 25 dissected mosquitoes on day 14 p.i., and sporozoites were counted similarly. Mosquito midguts were stained with mercurochrome, and oocyst diameters were measured using NIS-Elements D version 3.0 software with a 40× objective on a Nikon 510 microscope fitted with a Nikon DS-Ri1 digital camera.

**Fluorescence analysis of parasites expressing HA-tagged gene products.** Procedures for gene tagging were as described above. Parasite samples of different developmental stages were prepared differently (see the supplemental material).

**Statistical analysis.** The Mann-Whitney or two-sided t test (oocyst and sporozoite counts) was used for statistical tests. All data presented were from at least three independent experiments (repeats).

**Data availability.** All the data are provided in the main text and figures and the supplemental material accompanying the article.

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**SUPPLEMENTAL MATERIAL**

Supplemental material for this article may be found at [https://doi.org/10.1128/mBio.01986-17](https://doi.org/10.1128/mBio.01986-17).

**TEXT S1**, DOCX file, 0.02 MB.

**FIG S1**, TIF file, 6 MB.

**FIG S2**, TIF file, 4 MB.

**FIG S3**, TIF file, 3.9 MB.

**FIG S4**, TIF file, 2.9 MB.

**FIG S5**, TIF file, 3 MB.

**FIG S6**, TIF file, 3.1 MB.

**FIG S7**, TIF file, 2.6 MB.

**TABLE S1**, XLSX file, 0.01 MB.

**TABLE S2**, DOCX file, 1 MB.

**TABLE S3**, XLS file, 0.1 MB.

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REFERENCES

1. Cowman AF, Healer J, Maranapa D, Marsh K. 2016. Malaria: biology and disease. Cell 167:610–624. https://doi.org/10.1016/j.cell.2016.07.055.

2. Hall N, Karras M, Raine JD, Carlton JM, Kooij TW, Berriman M, Flores L, Janssen CS, Pain A, Christophides GK, James K, Rutherford K, Harris B, Harris D, Churcher C, Quail MA, Ormond D, Doggett J, Trueman HE, Mendoza J, Bidwell SL, Rajandream MA, Carucci DJ, Yates JR III, Kafatos FC, Janse CJ, Barrell B, Turner CM, Waters AP, Sinden RE. 2005. A comprehensive survey of the Plasmodium life cycle by genomic, transcriptomic, and proteomic analyses. Science 307:82–86. https://doi.org/10.1126/science.1103717.

3. Bozdech Z, Llinàs M, Pulliam BL, Wong ED, Zhu J, DeRisi JL. 2003. The transcriptome of the intraerythrocytic developmental cycle of Plasmodium falciparum. PLoS Biol 1:e5. https://doi.org/10.1371/journal.pbio.0000005.

4. Dietz KJ, Vogel MO, Viehhauser A. 2010. AP2/EREBP transcription factors are part of gene regulatory networks and integrate metabolic, hormonal and environmental signals in stress acclimation and retrograde signaling. Prototlasma 245:3–14. https://doi.org/10.1007/s00709-010-0142-8.

5. Phukan UJ, Jeena GS, Tripathi V, Shukla RK. 2017. Regulation of apetala2/ethylene response factors in plants. Front Plant Sci 8:150. https://doi.org/10.3389/fpls.2017.00150.

6. Jofuku KD, den Boer BG, Van Montagu M, Okamura JK. 1994. Control of Arabidopsis flower and seed development by the homeotic gene APETALA2. Plant Cell 6:1211–1225. https://doi.org/10.1105/tpc.6.9.1211.

7. Balaji S, Babu MM, Iyer LM, Aravind L. 2005. Discovery of the principal regulators controlling the AP2 domain of APETALA2 defines a large new family of DNA binding proteins. Nucleic Acids Res 33:3994–4006. https://doi.org/10.1093/nar/gki709.

8. Finn RD, Tate J, Mistry J, Coggill PC, Sammut SJ, Holz HR, Cerig C, Forlund K, Eddy SR, Sonnhammer EL, Bateman A. 2008. The Pfam protein families database. Nucleic Acids Res 36:D281–D288. https://doi.org/10.1093/nar/gkm960.

9. Modrzynska K, Pfander C, Chappell L, Yu L, Suarez C, Dundas K, Gomes AP, Newbold CI, Pain A, Berriman M, Janse CJ, Waters AP. 2014. A transcriptional switch underlies commitment to sexual development in Plasmodium falciparum telomeres. Cell Microbiol 16:12746. https://doi.org/10.1111/cmi.12742.

10. Zhang C, Xiao B, Jiang Y, Zhao Y, Li Z, Gao H, Ling Y, Wei J, Li S, Lu M, Su XZ, Cui H, Yuan J. 2014. Efficient editing of malaria parasite genome using the CRISPR/Cas9 system. mBio 5:e01414–14. https://doi.org/10.1128/mBio.01414–14.

11. Iwanaga S, Kaneko I, Kato T, Yuda M. 2012. Identification of an AP2-family protein that is critical for malaria liver stage development. PLoS One 7:e47557. https://doi.org/10.1371/journal.pone.0047557.

12. Flueck C, Barfai R, Niederwieser I, Wittmer K, Alako BT, Moes S, Bozdech Z, Jenoe P, Stunnenberg HG, Voss TS. 2010. A major role for the Plasmodium falciparum ApiAP2 protein PISP2 in chromosome end biology. PLoS Pathog 6:e1000784. https://doi.org/10.1371/journal.ppat.1000784.

13. Santos JM, Josling G, Ross P, Joshi P, Orchard L, Campbell T, Schieler A, Cristea IM, Llinàs M. 2017. Red blood cell invasion by the malaria parasite is coordinated by the PfAP2-1 transcription factor. Cell Host Microbe 21:731–741.e10. https://doi.org/10.1016/j.chom.2017.05.006.

14. Iwanaga S, Kaneko I, Kato T, Yuda M. 2012. Identification of an AP2-family protein that is critical for malaria liver stage development. PLoS One 7:e47557. https://doi.org/10.1371/journal.pone.0047557.

15. Pradhan S, Bongard T, Bakharev A, Villalonga B, Jones A, Fernández-Moreira V, Schäfer D, Reiter T, Wirth T, Yuda M. 2015. Global transcriptional profiling of the Plasmodium falciparum liver stage infected human liver. mBio 6:e01986–17. https://doi.org/10.1128/mBio.01986–17.

16. Yuda M, Iwanaga S, Kaneko I, Kato T, Yuda M. 2012. Identification of an AP2-family protein that is critical for malaria liver stage development. PLoS One 7:e47557. https://doi.org/10.1371/journal.pone.0047557.

17. Yuda M, Iwanaga S, Shigenobu S, Kato T, Kaneko I. 2010. Transcription factor AP2-Sp and its target genes in malarial sporozoites. Mol Microbiol 75:854–863. https://doi.org/10.1111/j.1365-2958.2009.07005.x.

18. Iwanaga S, Kaneko I, Kato T, Yuda M. 2012. Identification of an AP2-family protein that is critical for malaria liver stage development. PLoS One 7:e47557. https://doi.org/10.1371/journal.pone.0047557.

19. Flueck C, Barfai R, Niederwieser I, Wittmer K, Alako BT, Moes S, Bozdech Z, Jenoe P, Stunnenberg HG, Voss TS. 2010. A major role for the Plasmodium falciparum ApiAP2 protein PISP2 in chromosome end biology. PLoS Pathog 6:e1000784. https://doi.org/10.1371/journal.ppat.1000784.