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RESEARCH ARTICLE

# 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

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*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 ookinetes. The motile ookinetes 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 ookinete 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-I, 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 ookinetes 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 florescent 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 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

ring/trophozoite stages was similar to the level of gametocytemia (3.2%) observed for this parasite. We also tagged the PbAP2-G protein in *P. berghei* and again observed expression in the ring or early trophozoite stage but not in the schizont stage (Fig. S2F). The lack of expression in the schizonts (or expression at an undetectable level) of both *P. yoelii* and *P. berghei* suggests that these rodent parasites may have a sexual developmental process different from that of *P. falciparum*, which expresses PfAP2-G in the schizont stage (13). The developmental and morphological characteristics of the Δ*Pyap2-g* and *Pyap2-gΔ/gfp* parasites were similar to those reported for *P. falciparum* and *P. berghei*, confirming the essential role of *Pyap2-g* in *P. yoelii* gametocyte development. Similarly, parasites with disrupted *Pyap2-g2* had greatly reduced numbers of gametocytes and oocysts (Fig. S2G and H), although some gametocytes were observed in the *Pyap2-g2* KO parasite. The results show that PyAP2-G2 is critical for gametocyte development in *P. yoelii* and confirm a conserved evolutionary role for ApiAP2-G2 in *Plasmodium* gametocyte development.

Our analyses also identified a third gene (PY17X_1417400) that plays an important role in gametocyte development, and we therefore call the gene *Pyap2-g3*. The ortholog of this gene is resistant to genetic disruption in *P. berghei* (9), and its role in parasite development has not been investigated previously, although its putative function in gametocytogenesis was implicated in a previous study of *P. falciparum* parasites (24, 25). Here, we successfully disrupted the *Pyap2-g3* gene and showed that disruption of this gene significantly reduced the numbers of 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. S3A), ookinete conversion (Fig. S3B), the numbers of oocysts (Fig. S3C) or SG sporozoites (Fig. S3D). 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. S3E). 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. 2A), exflagellation (Fig. 2B), ookinete conversion, and morphology (Fig. 2C and D) but had significantly reduced ookinete motility (Fig. 2E). Parasites without this gene also produced no day 4 and day 8 oocysts (Fig. 2F) or SG sporozoites (Fig. 2G). Because it affects ookinete motility, leading to the absence of oocysts, and because PBANKA_1363700, the ortholog of PY17X_1369400, was previously designated *Pyap2-o4* (9), we call this gene *Pyap2-o5*. We also tagged this gene with the sequence encoding 6×HA and observed protein expression using IFA. PyAP2-O5 protein was again expressed mainly in the nucleus and weakly in the cytoplasm of some young schizonts and strongly expressed in the nuclei of male and female gametocytes, female gametes/zygotes, and retort ookinetes but absent (or undetectable) in mature ookinetes (Fig. 2H). To evaluate the parasite development in early developmental stages in the mosquito, we disrupted the *Pyap2-o5* gene in a parasite clone that was engineered to express mCherry-tagged sexual-specific protein P28 (26). The mCherry-tagged-P28-expressing parasite had gametocyte and oocyst developmental phenotypes similar to those of parasites with or without the *Pyap2-o5* gene, suggesting that the mCherry labeling of P28 did not change the gametocyte or

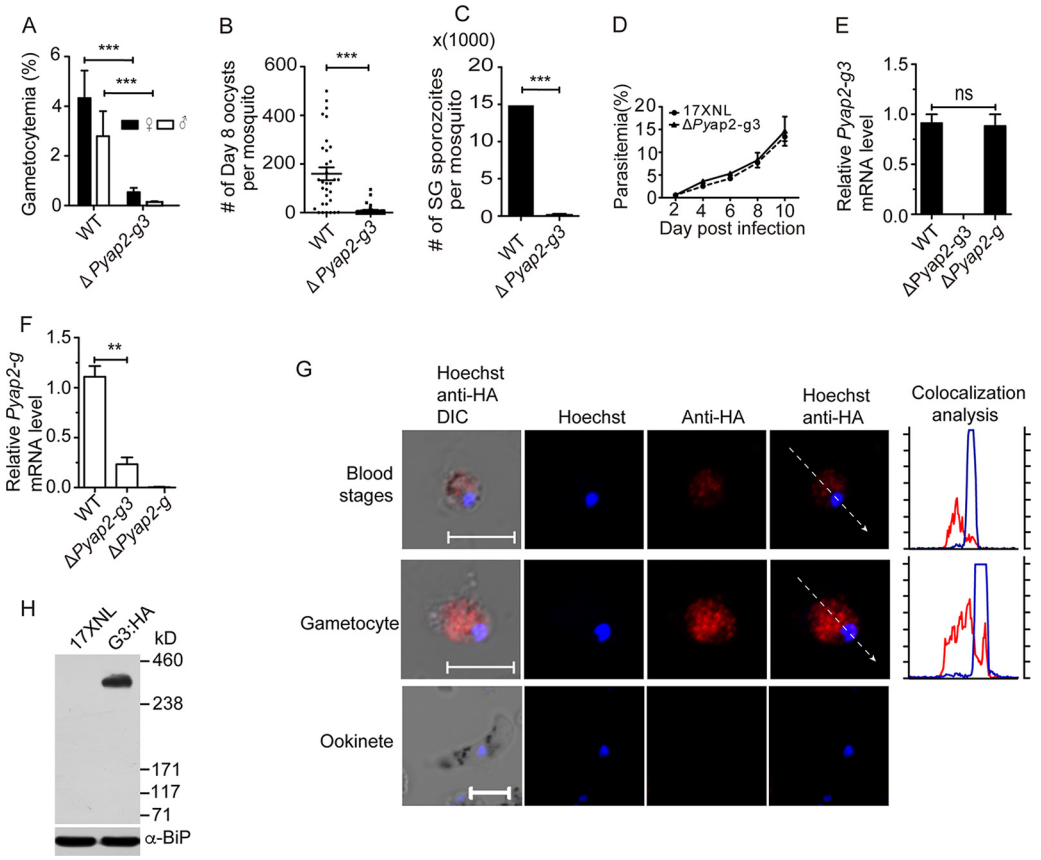


**FIG 1** Functional characterization of a gene critical for gametocyte development. (A) Gametocytemia was determined in Giemsa-stained blood films on day 3 p.i. Compared with wild-type (WT) 17XNL parasites, the *Pyap2-g3* KO parasites produced significantly fewer male and female gametocytes. Mean values and standard errors of the means (SEM), shown by error bars, were calculated from the results for three infected mice. (B) Total numbers of oocysts per mosquito on day 8 postfeeding with WT or *Pyap2-g3* KO parasites. Mean values and SEM were calculated from the results for 30 mosquito midguts. (C) Numbers of salivary gland (SG) sporozoites per infected mosquito on day 14 postfeeding. Mean values and SEM were calculated from the results of three independent experiments with 20 to 25 mosquitos each. (D) Levels of parasitemia caused by WT and *Pyap2-g3* KO parasites on days 2 to 10 postinfection. (E and F) Relative transcript levels in blood stages of WT, *Pyap2-g3* KO, or *Pyap2-g* KO parasites detected using quantitative reverse transcription (qRT)-PCR; signals were normalized against those for the 18S rRNA gene. The results are representative of two independent *Pyap2-g3* KO clones. (A to C and E to F) The results are representative of three independent experiments. (D) The data are representative of three mice. *P* values were determined using two-sided unpaired *t* test (B and C) or Mann-Whitney test (A and E to F). **, $P < 0.01$; ***, $P < 0.001$. (G) Immunofluorescence assay (IFA) of PyAP2-G3 protein expression in various developmental stages detected using anti-HA antibody (rabbit monoclonal antibody 3724; Cell Signaling Technology, Inc., Danvers, MA). DIC, differential interference contrast. Scale bar = 5 $\mu$M. (H) Detection of HA-tagged PyAP2-G3 expressed in lysates of blood stages. G3:HA, 6×HA-tagged PyAP2-G3. Anti-BiP antibody was included as the protein loading control.

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 oocysts (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

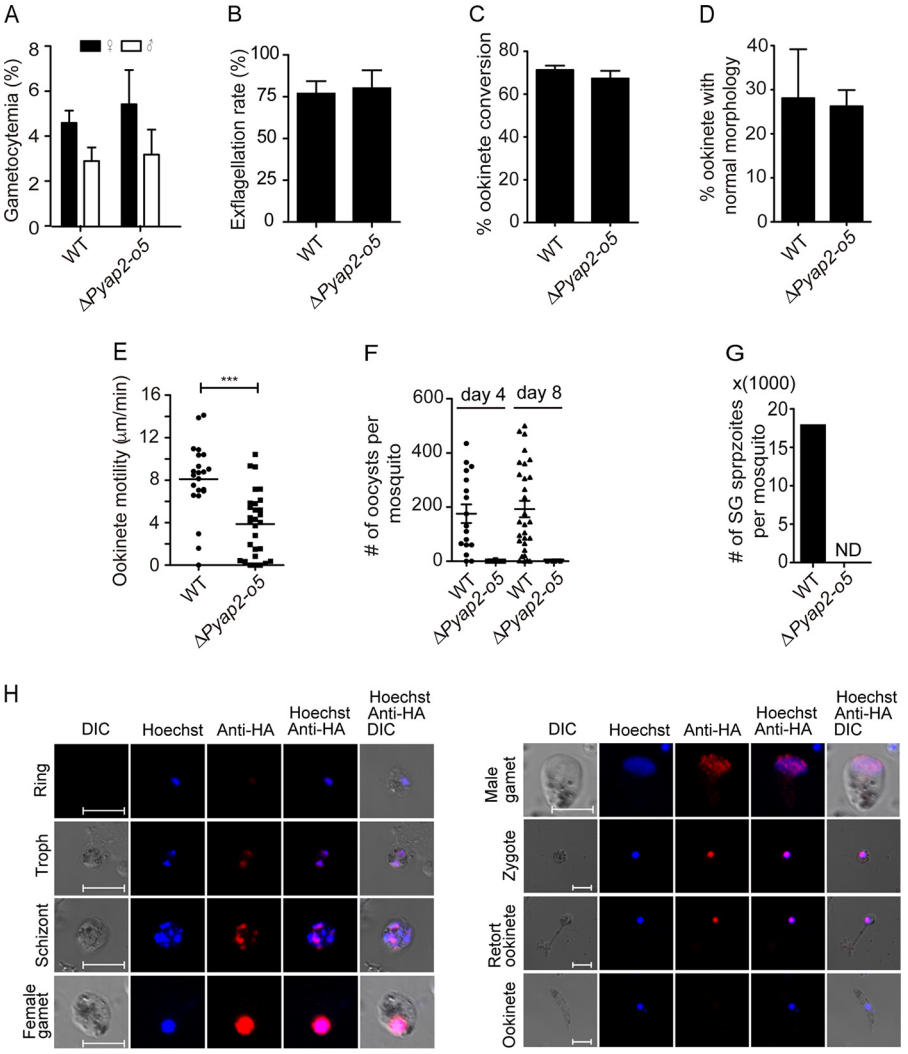


**FIG 2** Functional characterization of a new PyApiAP2 gene affecting ookinete motility and oocyst development. (A to D) Male and female gametocytemia on day 3 postinfection (A), exflagellation rate (B), ookinete conversion rate at 24 h (C), and ookinete morphology (D) of WT and *Pyap2-o5* KO parasites. Parasites were Giemsa stained and counted under a microscope. Mean values and SEM were calculated from the results for three infected mice or three independent experiments. The ookinete conversion rate is the number of retort and mature ookinetes divided by the number of female gametocytes in the same number of RBCs. (E) Motility of mature ookinetes measured on Matrigel. The paths of mature ookinetes were measured using ImageJ. Mean values were calculated from the results for 20 ookinetes (two-sided unpaired *t* test). ***, $P < 0.001$. (F) Total numbers of oocysts per mosquito at 4 and 8 days postfeeding. Mean values were calculated from the results for 30 mosquito midguts. (G) Numbers of salivary gland (SG) sporozoites per infected mosquito on day 14 postfeeding. (H) Immunofluorescence assay (IFA) of PyAP2-O5 protein expression in various developmental stages detected using anti-HA antibody. Scale bar = 5 $\mu$M. The data are representative of three independent experiments.

(Fig. 3F). 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

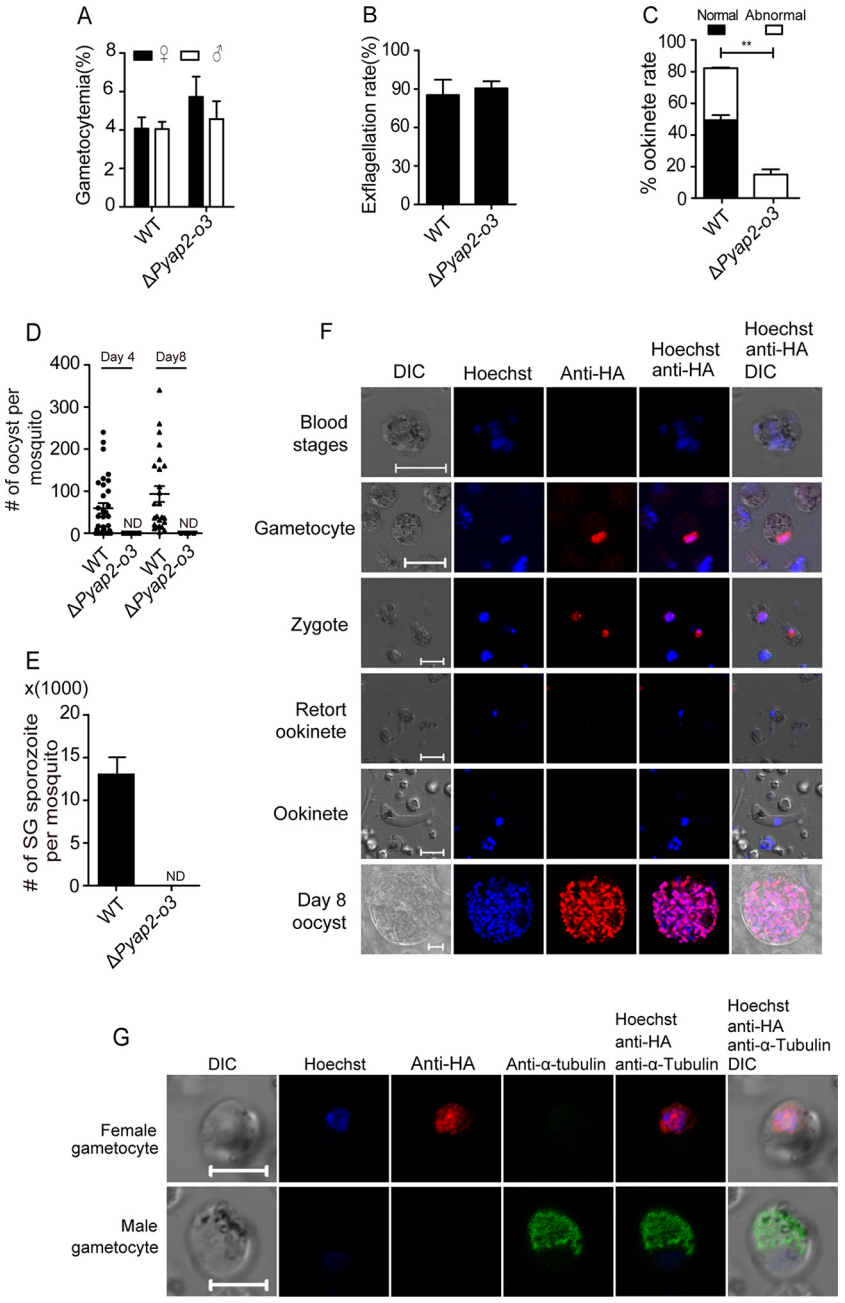


**FIG 3** Phenotypic and expression analyses of *Pyap2-o3* in blood and mosquito stages. (A and B) Male and female gametocytemia (A) and exflagellation rate (B) from blood samples on day 3 postinfection of WT or *Pyap2-o3* KO parasites. (C) Ookinete conversion rate (normal, mature ookinete; abnormal, 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 $\mu$M. (G) IFA images of male and female gametocytes double stained with anti-HA and anti-$\alpha$-tubulin antibodies. Scale bar = 5 $\mu$M. 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*

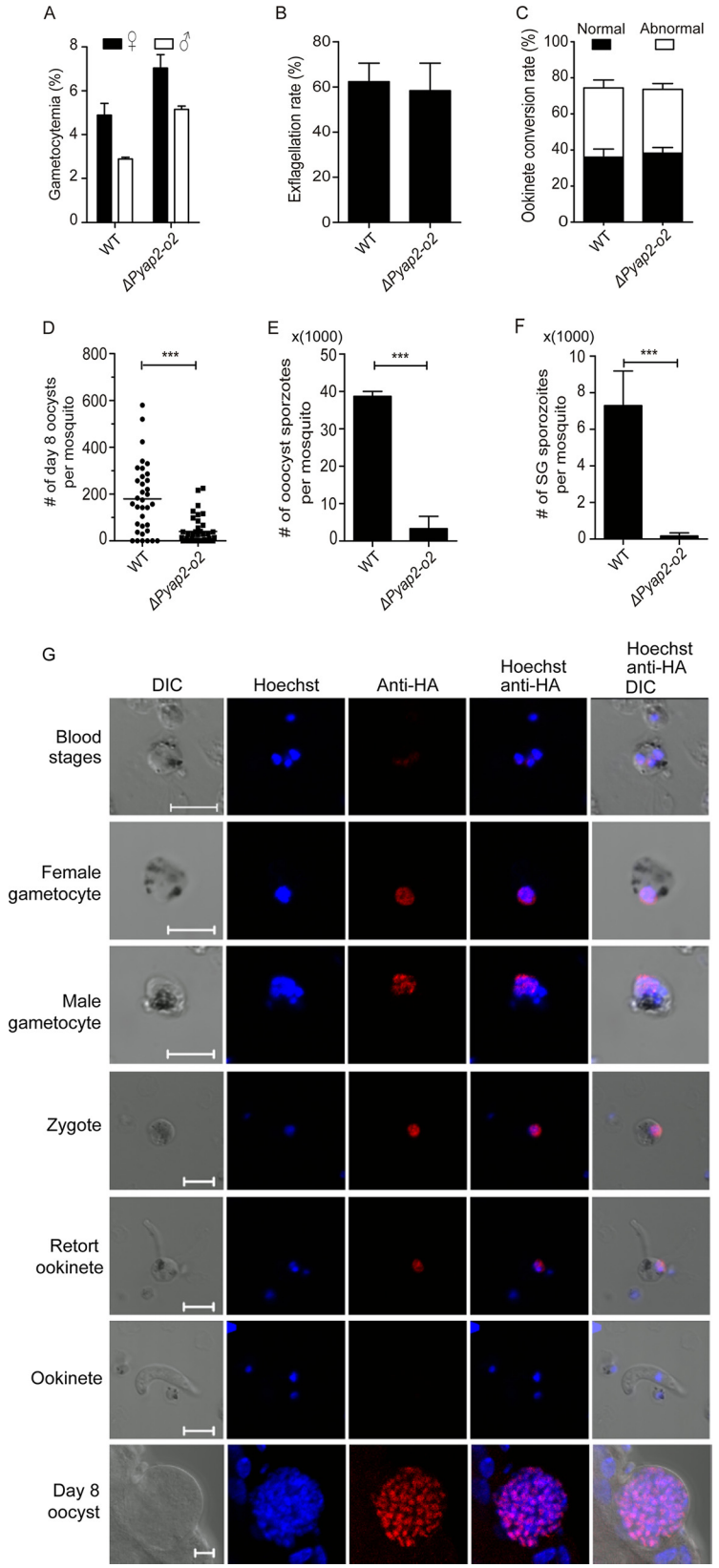


**FIG 4** Characterization of *Pyap2-o2* function and expression in blood and mosquito stages. (A to C) Male and female gametocytemia on day 3 postinfection (A), exflagellation rate (B), and ookinete conversion rate (C) from 24-h *in vitro* ookinete cultures of WT or *Pyap2-o2* KO parasites. (D) Numbers of day 8 oocysts per mosquito. (E and F) Numbers of day 12 oocyst sporozoites (E) or day 14 salivary gland (SG)

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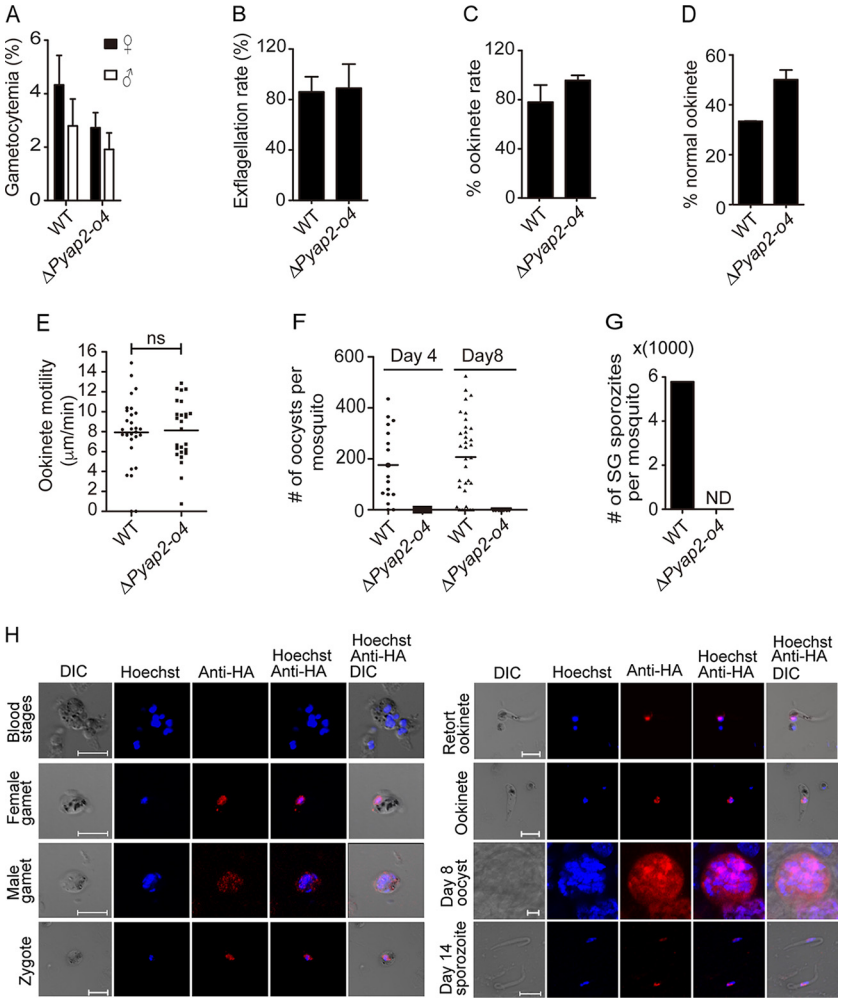



**FIG 5** Characterization of function and expression of *Pyap2-o4* in blood and mosquito stages. (A to E) Male and female gametocytemia on day 3 postinfection (A), exflagellation rate of male gametocytes (B), ookinete conversion rate (C), ookinete morphology (D), and ookinete motility (E) from 24-h *in vitro* ookinete cultures of WT and *Pyap2-o4* KO parasites. (F) Numbers of day 4 and day 8 oocysts per mosquito postfeeding of WT or *Pyap2-o4* KO parasites. (G) Numbers of day 14 salivary gland (SG) sporozoites 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. (H) 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

**FIG 4** Legend (Continued)
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* values were determined by the two-sided unpaired *t* test. ***, $P < 0.001$. (G) Immunofluorescence assay (IFA) of PyAP2-O2 protein expression in various developmental stages, detected using anti-HA antibody. Scale bar = 5 $\mu$M. The data are representative of three independent experiments or three mice.

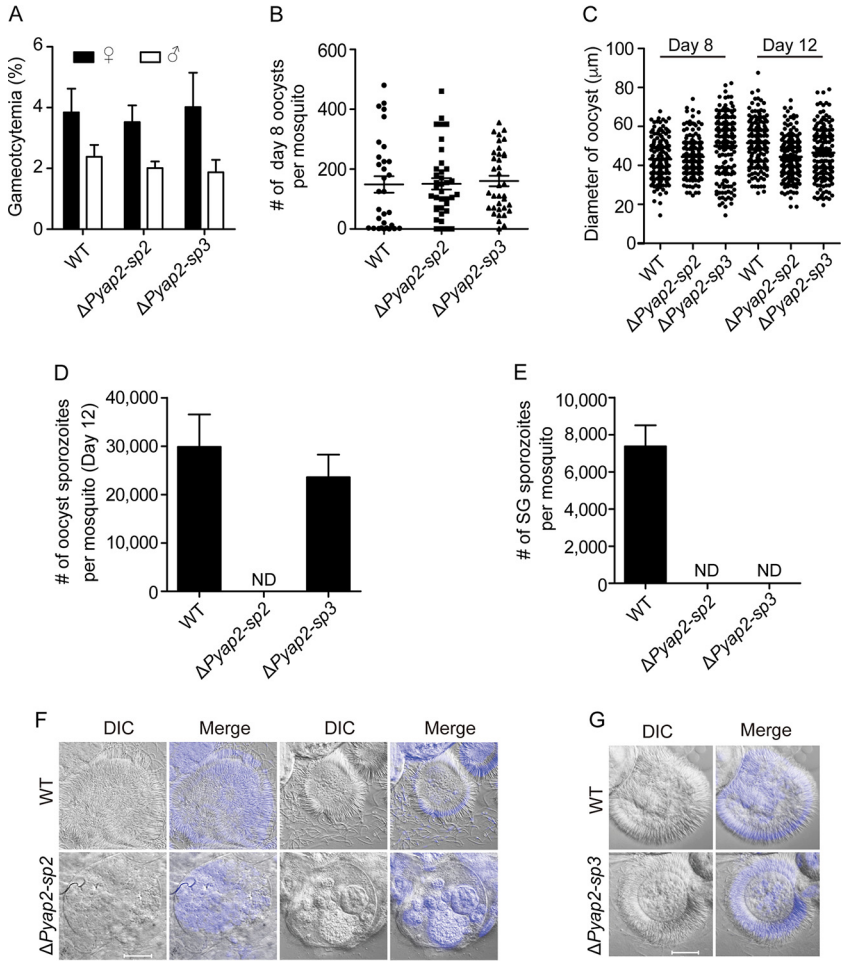



**FIG 6** Functional and phenotypic characterization of *Pyap2-sp2* and *Pyap2-sp3* in blood and mosquito stages. (A and B) Male and female gametocytemia on day 3 postinfection (A) and numbers of oocysts per mosquito on day 8 postfeeding (B) of WT, *Pyap2-sp2* KO, or *Pyap2-sp3* KO parasites. (C) Diameters of day 8 and day 12 oocysts from midguts of mosquitos 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 $\mu$m.

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.

## 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.

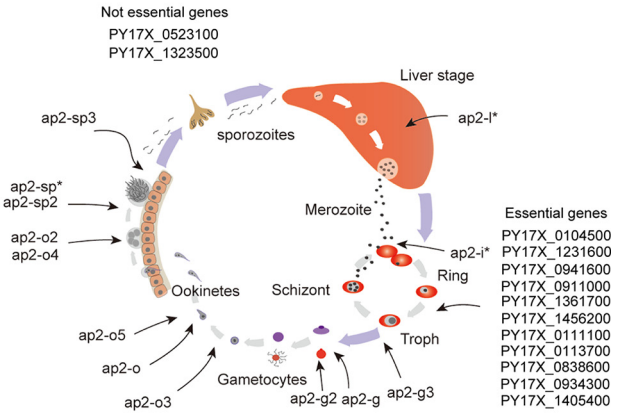
We also attempted to tag all 12 genes that affect the development of nonerythrocytic stages with sequences encoding 6×HA or mCherry to study their protein expression. We were able to detect expressed tagged proteins from seven genes (*Pyap2-g*, *Pyap2-g3*, *Pyap2-o*, *Pyap2-o2*, *Pyap2-o3*, *Pyap2-o4*, 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.

A

| Gene ID/*P.y.* | Gene ID/*P.f.* | Gene ID/*P.b.* | Name | AS1 | Gam | Exf. | Ook | Ooc | Oo. spz | SG spz | AS2 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| PY17X_1440000 | PF3D7_1222600 | PBANKA_1437500 | ap2-g | | | | | | | | |
| PY17X_1036700 | PF3D7_1408200 | PBANKA_1034300 | ap2-g2 | | | | | | | | |
| PY17X_0907300 | PF3D7_1143100 | PBANKA_0905900 | ap2-o | | | | | | | | |
| PY17X_1017000 | PF3D7_1429200 | PBANKA_1015500 | ap2-o3 | | | | | | | | |
| PY17X_1369400 | PF3D7_1350900 | PBANKA_1363700 | ap2-o4 | | | | | | | | |
| PY17X_1235000 | PF3D7_0516800 | PBANKA_1231600 | ap2-o2 | | | | | | | | |
| PY17X_1003200 | PF3D7_0404100 | PBANKA_1001800 | ap2-sp2 | | | | | | | | |
| PY17X_1123200 | PF3D7_0622900 | PBANKA_1121800 | ap2-sp3 | | | | | | | | |
| PY17X_1323500 | PF3D7_1456000 | PBANKA_1319700 | n | | | | | | | | |
| PY17X_1417400 | PF3D7_1317200 | PBANKA_1415700 | ap2-g3 | | | ND | | | | | |
| PY17X_1317000 | PF3D7_1449500 | PBANKA_1313200 | ap2-o5 | | | | | | | | |
| PY17X_0523100 | PF3D7_0420300 | PBANKA_0521700 | n | | | | | | | | |
| PY17X_0113700 | PF3D7_0613800 | PBANKA_0112100 | n | | | | | | | | |
| PY17X_0838600 | PF3D7_0934400 | PBANKA_0835200 | n | | | | | | | | |
| PY17X_0934300 | PF3D7_1115500 | PBANKA_0932300 | n | | | | | | | | |
| PY17X_1405400 | PF3D7_1305200 | PBANKA_1403700 | n | | | | | | | | |
| PY17X_0104500 | PF3D7_0604100 | PBANKA_0102900 | n | | | | | | | | |
| PY17X_1231600 | PF3D7_0802100 | PBANKA_1228100 | n | | | | | | | | |
| PY17X_1209100 | PF3D7_1007700 | PBANKA_1205900 | ap2-i | | | | | | | | |
| PY17X_0941600 | PF3D7_1107800 | PBANKA_0939100 | n | | | | | | | | |
| PY17X_0911000 | PF3D7_1139300 | PBANKA_0909600 | n | | | | | | | | |
| PY17X_1361700 | PF3D7_1342900 | PBANKA_1356000 | n | | | | | | | | |
| PY17X_1456200 | PF3D7_1239200 | PBANKA_1453700 | n | | | | | | | | |
| PY17X_0111100 | PF3D7_0611200 | PBANKA_0109500 | n | | | | | | | | |
| PY17X_1334500 | PF3D7_1466400 | PBANKA_1329800 | ap2-sp | | | | | | | | |
| PY17X_0215800 | PF3D7_0730300 | PBANKA_0214400 | ap2-l | | | | | | | | |

Normal (50-100%)
Reduced (10-50%)
Severely reduced (<10%)
Completely absent

B




C

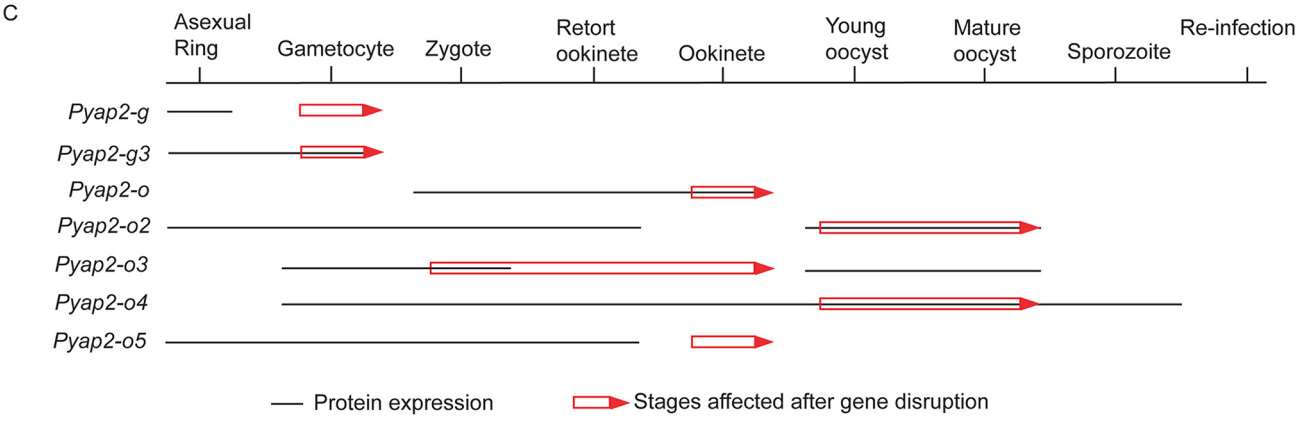



**FIG 7** Relationships between parasite developmental defects after selected PyApiAP2 gene disruption and protein expression profile. (A) Genetic disruption of the PyApiAP2 gene and its effects on parasite development in mice and mosquitoes. Genes in yellow are those that have been disrupted in *P. berghei* (9) and in this study; those in orange are genes that were successfully disrupted in this study but could not be disrupted in *P. berghei* (9); those in light green are genes that could not be disrupted in either *P. berghei* (9) or *P. yoelii* (this study); and those without color are genes that have been characterized in previous studies (12–18) and were not included in this study. AS1, asexual stages; Gam, gametocytes; Exf, exflagellation; Ook, ookinete; Ooc, oocyst; Oo. spz, oocyst sporozoite; SG spz, salivary gland sporozoite; AS2, second generation of asexual stages from mosquito feeding; ND, not determined. (B) Schematic of parasite life cycle with PyApiAP2 gene functional sites (causing developmental defect) indicated based on this and other previous studies. Nonessential genes are those that do not cause obvious developmental defects after gene disruption, whereas essential genes are those that could not be disrupted after at least 4 attempts. *, gene was not investigated in this study. (C) Protein expression profiles and parasite stages affected by gene disruption for the seven genes tagged with sequences encoding 6×HA or mCherry.

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

Compared with the results of the ApiAP2 gene disruption screen of *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 *P. berghei* but not for *P. yoelii*. One example of an APiAP2 gene having slightly different functions between *P. berghei* and *P. yoelii* is *ap2-o2*. Parasites without *Pbap2-o2* had greatly reduced ookinete numbers (9), whereas *Pyap2-o2*-deficient and WT parasites had similar ookinete conversion rates. *P. yoelii* and *P. berghei* are closely related parasites, and approximately 90% of the predicted proteins in rodent malaria parasites (*P. berghei*, *P. yoelii*, and *Plasmodium chabaudi*) have orthologs in primate malaria parasites (23, 29, 30), supporting evolutionarily conserved gene functions in *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 procedures 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 (32) 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 (26, 33) 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 50i 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.

## SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at 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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C.Z., Z.L., H.C., H.G., and C.L. performed experiments; Y.J., Z.Y., X.W., and S.Z. performed phenotype screening of mutants; J.Y. designed the study; and Y.J. and X.-Z.S. analyzed the data and wrote the manuscript.

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