*Pf*CDPK1 is critical for malaria parasite gametogenesis and mosquito infection

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Efforts to knock out *Plasmodium falciparum* calcium-dependent protein kinase 1 (*Pf*CDPK1) from asexual erythrocytic stage have not been successful, indicating an indispensable role of the enzyme in asexual growth. We recently reported generation of a transgenic parasite with mutant CDPK1 [Bansal A, et al. (2016) *MBio* 7:e02011-16]. The mutant CDPK1 (T145M) had reduced activity of transphosphorylation. We reasoned that CDPK1 could be disrupted in the mutant parasites. Consistent with this assumption, CDPK1 was successfully disrupted in the mutant parasites using CRISPR/Cas9. We and others could not disrupt *Pf*CDPK1 in the WT parasites. The CDPK1 KO parasites show a slow growth rate compared with the WT and the CDPK1 T145M parasites. Additionally, the CDPK1 KO parasites show a defect in both male and female gametogenesis and could not establish an infection in mosquitoes. Complementation of the KO parasite with full-length *Pf*CDPK1 partially rescued the asexual growth defect and mosquito infection. Comparative global transcriptomics of WT and the CDPK1 KO schizonts using RNA-seq show significantly high transcript expression of gametocyte-specific genes in the CDPK1 KO parasites. This study conclusively demonstrates that CDPK1 is a good target for developing transmission-blocking drugs.

*Pf*CDPK1 | plasmodium | compensation | gametocytes | mosquito

Protein kinases are being actively pursued as targets for drug development against human malaria. Calcium-dependent protein kinases (CDPKs) play critical roles at various stages of *Plasmodium* development and importantly are not expressed in humans. These characteristics qualify CDPKs as good targets for drug development. *Plasmodium falciparum* contains seven members of the CDPK family, CDPK1 to CDPK7. CDPK1 is the most widely studied member of the *P. falciparum* CDPK family. CDPK1 contains N-terminal motifs that play an important role in the membrane anchoring and the correct localization of CDPK1 in the parasite (1).

Recombinant *Pf*CDPK1 was shown to phosphorylate components of the motor complex, by an in vitro phosphorylation assay (2). A pharmacological inhibitor, purfalcamine, inhibited egress of merozoites from mature segmented schizonts (3). Treatment of parasites with purfalcamine was later demonstrated to block the discharge of micronemes (4) and subsequent invasion of host RBCs (4). Overexpression of the *Pf*CDPK1 junction domain implicated the importance of CDPK1 in late schizogony (5). Protein kinase G (PKG)-dependent phosphorylation of *Pf*CDPK1 in the parasite led to its preferential localization at the apical pole in schizonts and free merozoites (6). However, the physiological role of phosphorylated CDPK1 and its apical localization is not well understood. *Pf*CDPK1 was shown to phosphorylate the regulatory subunit of cAMP-dependent protein kinase A (*Pf*PKA-R) along with proteins of the inner membrane complex, further substantiating its role in invasion of RBCs and normal asexual growth using a conditional knockout technique (7). Failure to knock out CDPK1 from the asexual stages of *P. falciparum* by independent laboratories suggested a critical role of the enzyme at this stage of the parasite life cycle (3, 8). With the understanding of an indispensable role of CDPK1 in the asexual stage of the parasite life cycle, it is being pursued as a drug target for the treatment of clinical malaria.

Transgenic parasites with mutant CDPK1 (CDPK1 T145M) were shown to exhibit increased sensitivity for compound 2 (C2) (9), a specific inhibitor of PKG (10), accompanied by changes in transcription of other kinases (9). These results suggested compensatory mechanisms in the mutant parasite for reduced activity of mutant CDPK1. We reasoned that compensatory mechanisms may allow the parasite to adapt for the loss of the CDPK1 gene.

In the present study, we show the successful disruption of the endogenous CDPK1 gene in the CDPK1 T145M parasite background using clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated protein 9 (Cas9) gene editing technique. Note that efforts to knock out CDPK1 in the WT NF54 parasites were not successful in three independent attempts. To capture the functions of CDPK1, we compared the CDPK1 KO parasites with the WT and not the CDPK1 T145M parasites. The CDPK1 KO parasites show slow asexual proliferation compared with the WT. Additionally, the CDPK1 KO parasites are defective in the formation of male and female gametes and fail to establish infection in mosquitoes. We conclusively show the functions of CDPK1 in the parasite life cycle by

Significance

We have shown in this study that the malaria parasite can rapidly evolve to adapt for loss of an "essential" kinase, *Pf*CDPK1. *Pf*CDPK1 could not be disrupted in the wild-type parasite. However, we were able to disrupt CDPK1 in the transgenic parasites adapted for reduced kinase activity of mutant *Pf*CDPK1. Strategic disruption of *Pf*CDPK1 highlights the importance of understanding the compensatory mechanisms, especially for targets belonging to multigene families. Our study unequivocally demonstrates that *Pf*CDPK1 is critical for mosquito infections and its disruption leads to defective gametogenesis. Our study also suggests involvement of CDPK1 in regulation of sexual stage-specific genes during the asexual proliferation. Targeting *Pf*CDPK1 may be a good strategy for developing transmission-blocking drugs.

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complementing the CDPK1 KO parasites with WT copy of full-length CDPK1.

Results

Disruption of Endogenous *Pf*CDPK1. We failed to knock out *Pf*CDPK1 in the WT parasites in three independent attempts. We successfully disrupted the endogenous *Pf*CDPK1 gene using CRISPR/Cas9 gene editing technique (Fig. 1 and *SI Appendix*, Fig. S1) in the CDPK1 T145M parasites (9). The oligo pair F1/R1 specifically amplified in WT and CDPK1 T145M parasites and not in the CDPK1 KO, yielding the expected product of 628 bp (*SI Appendix*, Fig. S1*B*). The WT and CDPK1 T145M parasites amplified a product of 1,797 bp while an amplicon of 3,726 bp was obtained with the CDPK1 KO clones using the primer pair F1/R2 (*SI Appendix*, Fig. S1*B*; primer sequences are provided in *SI Appendix*, Table S6), confirming substitution of the kinase domain of CDPK1 with the hDHFR cassette in the CDPK1 KO parasites.

We further confirmed the disruption of *Pf*CDPK1 by Western blot with late-asexual-stage parasites using specific antibodies against *Pf*CDPK1 (2). Protein bands of an expected molecular weight of ~61 kDa were observed in the WT and CDPK1 T145M parasites and not in the CDPK1 KO clones 31 and 32 (Fig. 1*A*). Antibodies against β actin, used as a loading control for the parasite lysate, are highlighted in all of the loaded lanes (Fig. 1*A*, *Bottom*). The disruption of CDPK1 was also confirmed by immunofluorescence assay (IFA) in the mature schizonts of CDPK1 KO. Schizonts and free merozoites of WT parasites showed typical peripheral localization of CDPK1 (*SI Appendix*, Fig. S1*C*). As expected, the CDPK1 KO schizont did not result in staining with anti-CDPK1 (*SI Appendix*, Fig. S1*C*). These results conclusively show successful disruption of endogenous CDPK1 gene and absence of the protein.

PfCDPK1 KO Parasites Grow More Slowly than the WT Parasites. The CDPK1 KO parasites (KO C31 and KO C32) grew more slowly than the WT and CDPK1 T145M parasites (Fig. 1 *B*, *i*), suggesting an important role of CDPK1 during asexual proliferation. The growth defect phenotype in *P. falciparum* CDPK1 KO parasites is in contrast to the knock-out of CDPK1 in *Plasmodium berghei* where its deletion did not result in any asexual growth defect (11), indicating either different functions of the homologs in the two species or different levels of dependence on CDPK1 with same functional role.

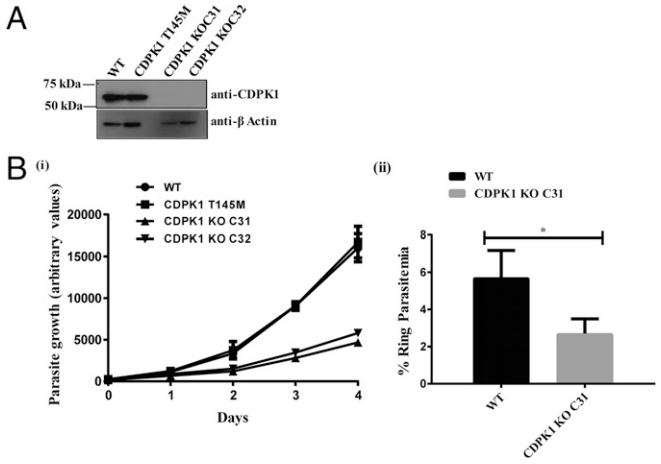

Fig. 1. The CDPK1 KO parasites show an asexual growth defect. (*A*) Analysis of CDPK1 protein expression in the CDPK1 KO parasites. A representative Western blot with late asexual stages of WT and CDPK1 T145M parasites, probed with specific antibodies against CDPK1 (anti-CDPK1) and anti-β actin. (*B*, *i*) The parasite growth (arbitrary values) on the *y* axis is plotted against days on the *x* axis for CDPK1 KO (clones 31 and 32), WT, and CDPK1 T145M parasites. The error bars represent SDs from two independent experiments conducted in quintuple. (*B*, *ii*) The graph show percent ring parasitemia on the *y* axis for the WT and CDPK1 KO C31 parasites. The error bars represent SDs from three independent biological experiments conducted in triplicate. **P* = 0.025, paired *t* test.

Previous studies suggested a role of CDPK1 during invasion of RBCs by the parasite (4, 7). We tested whether the growth defect in CDPK1 KO parasites could be due to a defect in invasion. For this study, an equal number of mature schizonts were incubated with fresh RBCs, and new invasions were evaluated through Giemsa smear after 8–10 h. The percent ring parasitemia in the CDPK1 KO parasites was significantly less compared with the WT (Fig. 1 *B*, *ii*; paired *t* test, $P = 0.025$). Although unruptured schizonts were similar in the CDPK1 KO and WT parasites, a more elaborate experimental design is required to rule out an egress defect in the CDPK1 KO parasites.

C2, a specific inhibitor of PKG (10), had significantly greater inhibition in growth of CDPK1 KO compared with the WT and the CDPK1 T145M parasites ($P < 0.0001$) (*SI Appendix*, Fig. S2). Moreover, CDPK1 T145M parasites showed greater sensitivity with C2 compared with the WT ($P = 0.002$) (*SI Appendix*, Fig. S2), consistent with the previous finding (9). The IC_{50} of C2 for WT, CDPK1 T145M, KO C31, and KO C32 parasites were 450 ± 30 (mean ± SEM), 326 ± 7.4, 129 ± 8.1, and 138 ± 3, respectively. These results suggest that the compensatory mechanisms resulting from CDPK1 disruption in the CDPK1 KO parasites are mediated through PKG activity.

***Pf*CDPK1 KO Parasites Are Defective in Gametogenesis.** The CDPK1 protein was expressed in the mature male and female gametocytes and gametes (*SI Appendix*, Fig. S3; also see *SI Appendix, Results*). The mature male and female gametocytes of CDPK1 KO parasites looked morphologically similar to the WT by IFA (*SI Appendix*, Fig. S4; also see *SI Appendix, Results*). The mature male and female gametocytes in WT round-up after induction and male gametes were seen exiting the residual body during the process of exflagellation (97%) (Fig. 2 and *SI Appendix*, Table S5). The numbers of exflagellation centers in the WT were 1.62–14.75 per field of view using a 40× objective (*SI Appendix*, Tables S1 and S2).

While the gametes of the WT looked normal, the gametes of CDPK1 KO were abnormal. The mature male gametocytes of CDPK1 KO round up after induction but did not exflagellate (100%) (*SI Appendix*, Table S5), except for 1% in live assay (*SI Appendix*, Table S1). The staining of α-tubulin II (green) in the WT and CDPK1 KO parasites showed either a diffuse pattern (3% and 93%, respectively) or flagellar structures (97% and 7%, respectively) in the male gametocytes after induction (Fig. 2 and *SI Appendix*, Table S5), suggesting that the KO parasites are defective in forming flagella. The rare abnormal exflagellations are reminiscent of the PPLP2 KO parasites (12), whereby the parasites were not able to exit the RBC and the flagella were seen as a "superflagellum" instead of eight individual flagella (Fig. 2). We observed that most of the mature female gametocytes of CDPK1 KO do not round up after induction (95%) (Figs. 2 and 3*A* and *SI Appendix*, Table S5). This is in contrast to the *Pf*CDPK2 KO, where the female gametocytes round up and exit the RBC membrane (13). Taken together, these results indicate that CDPK1 is critical for the formation of both male and female gametes.

The WT female gametes successfully exit the RBC after induction (91%) (absence of band 3, Fig. 3*A* and *SI Appendix*, Table S5). In contrast, the female gametes of CDPK1 KO remained inside the RBC (100%) (presence of band 3, Fig. 3*A*, cyan and *SI Appendix*, Table S5). The WT male gametes were seen in the exflagellation center devoid of RBC membrane (97%) (absence of band 3, Fig. 3*B* and *SI Appendix*, Table S5). In contrast, the RBC membrane around the male gametes of CDPK1 KO parasites was intact after the induction (100%) (presence of band 3, Fig. 3*B*, red). The WT resulted in 17, 116, and 295 exflagellation centers in 10, 16, and 20 fields of 40×

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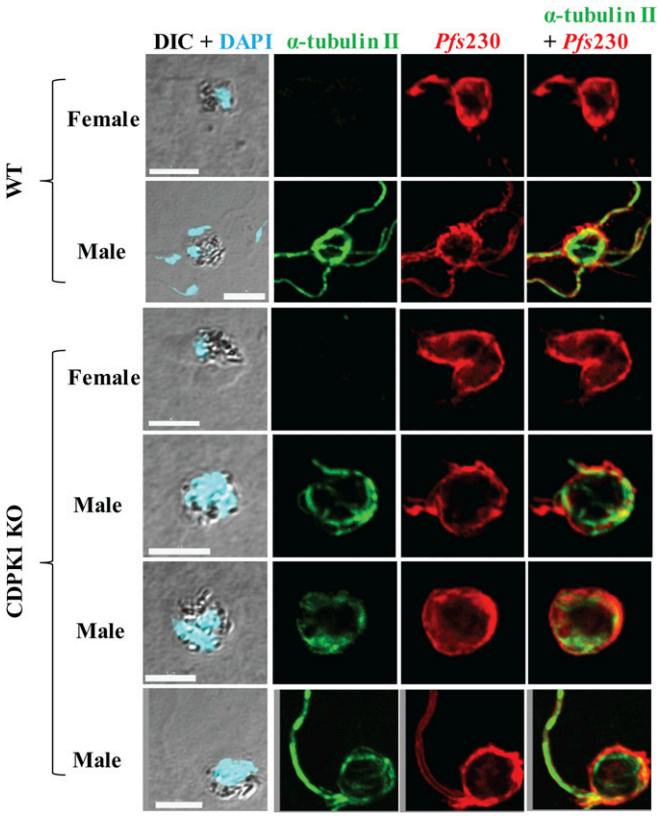

Fig. 2. CDPK1 KO parasites are defective in gametogenesis. Female and male gametes of WT and CDPK1 KO were stained for α-tubulin II (green), a male-specific marker, and *Pfs*230 (red). (Scale bars, 5 μm.) DIC, differential interference contrast.

objective, respectively, while only one exflagellation center was observed in CDPK1 KO parasites in a total of 100 fields (*SI Appendix*, Table S1).

***Pf*CDPK1 KO Parasites Do Not Infect Mosquitoes.** The CDPK1 KO C31 and C32 parasites used for mosquito infections contained 1.3–2.7% and 2.1–2.5% stage V gametocytes (*SI Appendix*, Table S1), respectively. The sex ratio of male to female gametocytes in the CDPK1 KO C31 was similar to the WT (*SI Appendix*, Table S3). However, the CDPK1 KO parasites failed to infect the mosquitoes as is evident by the absence of oocysts in the dissected midguts (Fig. 3*C* and *SI Appendix*, Table S1) while the WT parasites resulted in an infection rate of 75–81.8% (Fig. 3 and *SI Appendix*, Table S1). The median oocyst numbers in the mosquitoes infected with WT parasites were 2.5–3 (*SI Appendix*, Table S1). Taken together, these results confirm that *Pf*CDPK1 is critical for mosquito infection, likely due to its key role in gametogenesis.

Expression of *Pf*CDPK1 Rescues Defects in the CDPK1 KO C31 Parasites. We expressed the WT copy of full-length *Pf*CDPK1 with a C-terminal V5 tag under the ef1α promoter in the CDPK1 KO C31 parasites. The complemented parasites are denoted hereafter as CDPK1 KO+CDPK1V5. Western blot with asexual-stage parasites (36–48 h) using anti-CDPK1 antibodies detected protein of the desired molecular weight of ∼62.5 kDa in CDPK1 KO+CDPK1V5 and not in CDPK1 KO and the KO parasites with empty plasmid (CDPK1 KO+EP) (Fig. 4*A*). A similar-size band was detected in the CDPK1 KO+CDPK1V5 parasites by anti-V5 tag and anti-CDPK1 antibodies (Fig. 4*A*). The antibodies to β actin were used as a loading control for the parasite material (Fig. 4*A*). This result confirms expression of full-length CDPK1 in the CDPK1 KO+CDPK1V5 parasites.

The CDPK1 KO+CDPK1V5 parasites showed a modest increase in the asexual growth compared with the CDPK1 KO (Fig. 4*B* and *SI Appendix*, Fig. S5) in four independent experiments, and the difference in day 4 parasitemia was statistically significant (paired *t* test, $P = 0.0084$). The WT parasites with the episomal CDPK1 showed a modest decrease in the asexual proliferation compared with the WT parasites (Fig. 4*B* and *SI*

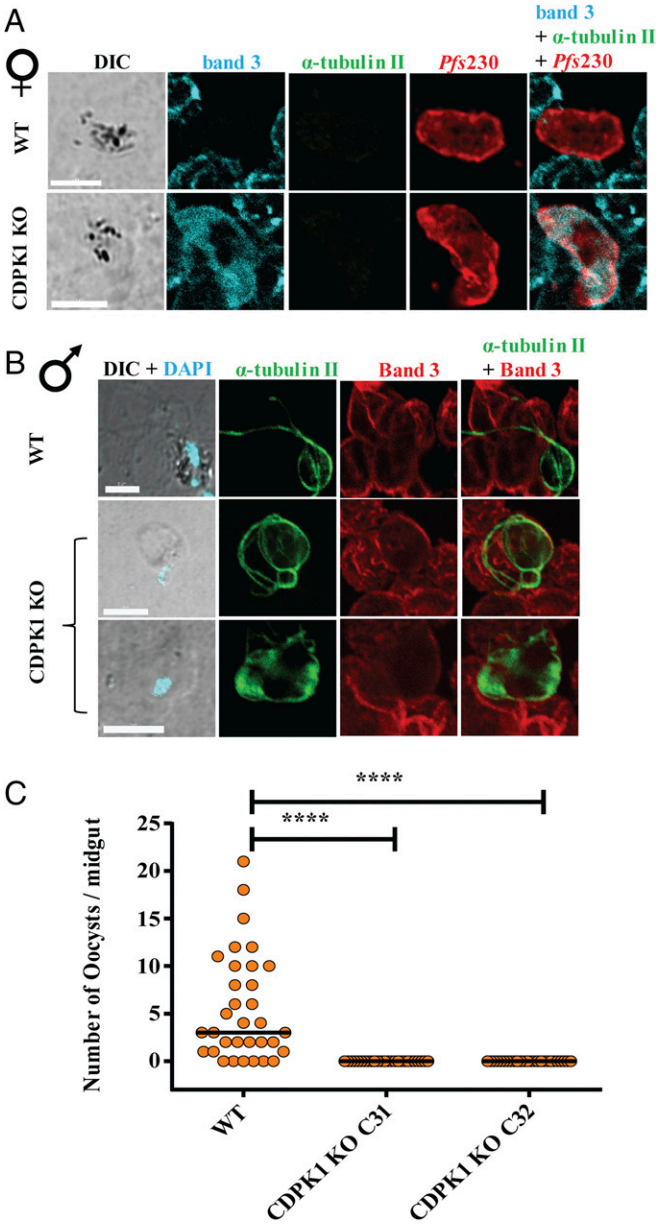

Fig. 3. The CDPK1 KO gametes are defective in exiting from the RBCs and fail to infect mosquitoes. (*A*) Mature female gametocytes of WT and CDPK1 KO after induction were stained for band 3 (cyan), an RBC surface marker, *Pfs*230 (red), and were negative for α-tubulin II (green). Nuclei stained with DAPI and DIC are shown. (Scale bars, 5 μm.) (*B*) Mature male gametocytes of WT and CDPK1 KO stained for α-tubulin II (green) and band 3 (red) after induction. (Scale bars, 5 μm.) (*C*) The CDPK1 KO parasites are unable to infect mosquitoes (also see *SI Appendix*, Table S1). The graph (experiment 1 of *SI Appendix*, Table S1) shows the number of oocysts/midgut in mosquitoes infected with WT, CDPK1 KO C31, and CDPK1 KO C32 parasites. Each orange circle represents an individual mosquito and the black horizontal lines represent the median. ****$P < 0.0001$. DIC, differential interference contrast.

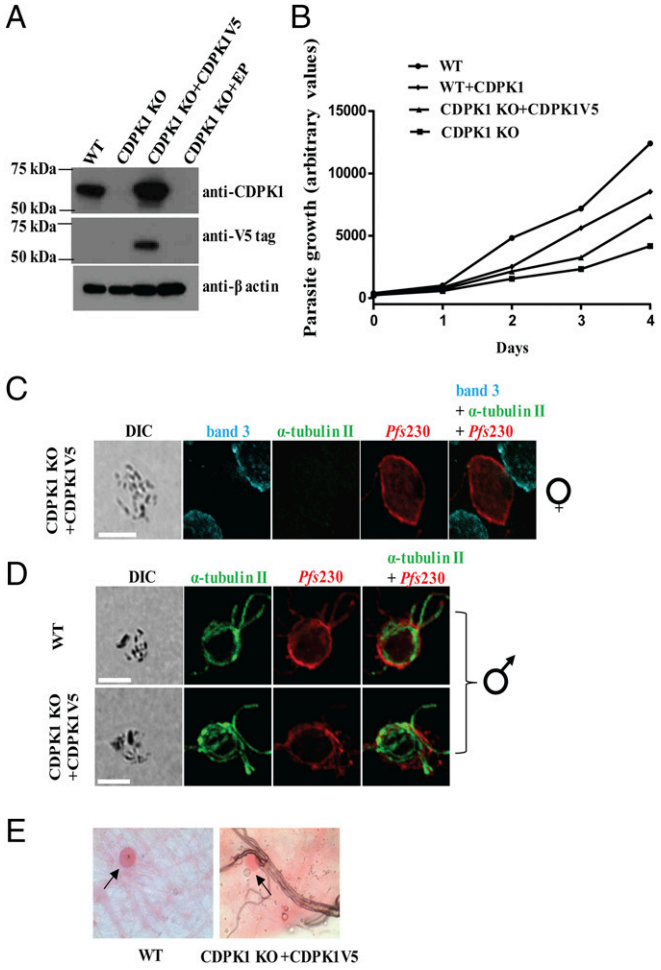

Fig. 4. Ectopic expression of CDPK1 in the KO parasites partially rescues the defects in the complemented parasites (CDPK1 KO+CDPK1V5). (*A*) Western blots with asexual stages (36-48 HPI) of WT, CDPK1KO, CDPK1 KO+CDPK1V5, and CDPK1 KO with empty plasmid (CDPK1 KO+EP) probed with sera against CDPK1(anti-CDPK1), V5-tag (anti-V5 tag), and β actin (anti-β actin). Molecular weights are indicated. (*B*) The graph shows asexual growth (arbitrary values) on the *y* axis plotted against number of days on the *x* axis for WT, CDPK1 KO, CDPK1 KO+CDPK1V5, and the WT parasites containing plasmid constitutively expressing CDPK1 (WT+CDPK1V5). The graph is plotted from five technical replicates and is a representative of four independent experiments (see *SI Appendix*, Fig. S4). (*C*) The female gamete of CDPK1 KO+CDPK1V5 stained for band 3 (cyan), an RBC surface marker, *Pfs*230 (red), and were negative for α-tubulin II (green). (*D*) Exflagellation centers stained for α-tubulin II (green) and *Pfs*230 (red) in the WT and CDPK1 KO+CDPK1V5 parasites are shown. (Scale bar, 5 μm.) (*E*) An oocyst is shown by a black arrow in the midgut of a mosquito infected with WT and CDPK1 KO+CDPK1V5 parasites. DIC, differential interference contrast.

Appendix, Fig. S5 *B* and *C*). This result suggests that overexpression of CDPK1 has an inhibitory effect on the parasite growth.

The female gametes of CDPK1 KO+CDPK1V5 round up and exit the RBC membrane (absence of band 3, Fig. 4*C*). Similarly, the male gametes of CDPK1 KO+CDPK1V5 were seen exiting the RBC membrane in a normal-looking exflagellation center as the WT (Fig. 4*D*). Moreover, exflagellation centers were readily observed in the CDPK1 KO+CDPK1V5 parasites in the live assay (*SI Appendix*, Table S2) and varied from 0.5 to 1 exflagellation per 40× objective. Importantly, the CDPK1 KO+CDPK1V5 parasites were able to establish infection in the mosquito as evidenced by the presence of oocysts in the dissected midguts (Fig. 4*E* and *SI Appendix*, Table S2). The percentage infection in mosquitoes fed with WT or CDPK1 KO+CDPK1V5 parasites ranged from 24.4 to 100% or 0 to 15%, respectively (*SI Appendix*, Table S2). Mosquitoes fed with the CDPK1 KO+EP parasites did not infect mosquitoes (*SI Appendix*, Table S2). These results show that the loss in the ability to infect mosquitoes in the CDPK1 KO parasites is due to CDPK1 disruption. Taken together, our results clearly demonstrate that CDPK1 is critical for mosquito infections and is important for the asexual growth of the parasite.

***Pf*CDPK1 Is Involved in Transcription Regulation of Gametocyte-Specific Genes.** It is important to note that CDPK1 KO parasites have 45–60% less invasion of RBCs compared with WT. Therefore, the loss of CDPK1 could only be partially compensated. In our RNA-seq analysis with mature schizonts [44–48 h postinvasion (HPI)] of the WT and CDPK1 KO parasites, we could not find a kinase or a phosphatase with a striking difference in expression in the CDPK1 KO parasites that could explain compensation for the lack of CDPK1 (Dataset S1). However, we found significant up-regulation (~2.4-fold) of raf kinase inhibitor (RKIP) in CDPK1 KO parasites. *Plasmodium* RKIP was shown to be a substrate of *Pf*CDPK1 and, in turn, decreased *Pf*CDPK1's transphosphorylation activity (14).

Interestingly, we found that early gametocyte-specific genes were highly up-regulated in the CDPK1 KO parasites (Fig. 5 and *SI Appendix*, Fig. S6 and Table S4). The topmost genes in this list include gametocyte development protein 1 (GDV1) and the transcription factor AP2-G. *Pf*GDV1 was shown to play a key role in early sexual-stage differentiation and its deletion led to loss of gametocytogenesis (15). AP2-G is a master regulator for commitment to sexual life cycle in *Plasmodium* (16, 17). Since the CDPK1 KO parasites showed increased transcript expression of AP2-G, we tested if CDPK1 KO parasites have increased sexual commitment. However, the sexual commitment of the asexual-stage parasites in CDPK1 KO was not significantly different from the WT (paired *t* test, $P = 0.27$) (*SI Appendix*, Fig. S7*B*), as estimated by percent *Pfs*16+ parasites after 30–34 HPI (*SI Appendix*, Fig. S7*A*).

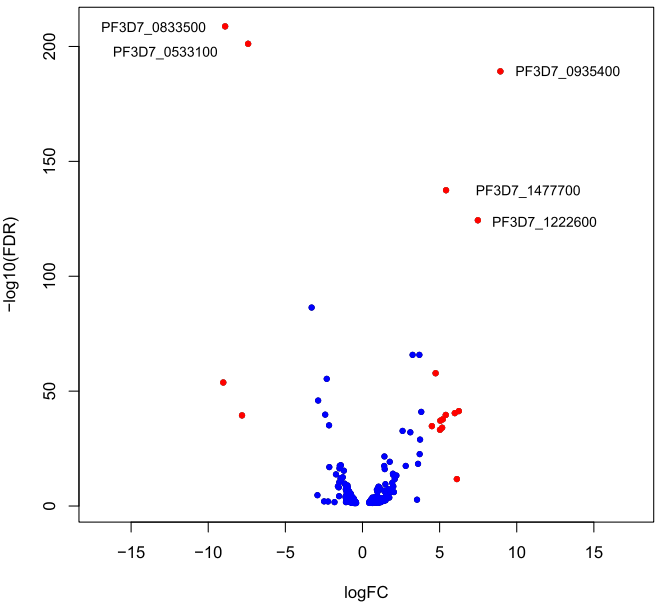

Fig. 5. A volcano plot showing up-regulated (*Right*) and down-regulated genes (*Left*) in the KO parasites relative to the WT. The red dots represent highly up-regulated and down-regulated genes. The graph shows the negative logarithm of FDR, −log10(FDR), on the *y* axis and the logarithm of FC, logFC, on the *x* axis from RNA-seq data from four independent biological replicates. FC, fold change; FDR, false discovery rate.

The sporozoite proteins, such as cell traversal protein for ookinetes and sporozoites (CelTOS) and sporozoite invasion-associated protein 1 (SIAP1), were found to increase in the CDPK1 KO parasites. *Pb*CelTOS was shown to be important for cell traversal activity of sporozoites and ookinetes (18). *Pb*SIAP1 was shown to play an important role in gliding locomotion and colonization of the salivary glands (19). We verified the RNA-seq expression data with real-time qPCR. Overall, the RNA-seq and qPCR data correlated well (*SI Appendix*, Fig. S6), except that cyclin-dependent kinase regulatory subunit (PF3D7_0923500) did not show an up-regulation in qPCR (*SI Appendix*, Table S4), contrary to modest up-regulation in RNA-seq. The expression of CDPK5 and CDPK6 showed a modest, significant increase by qPCR in CDPK1 KO (*SI Appendix*, Table S4). Taken together, the RNA-seq data suggest that CDPK1 may be involved in the transcription regulation of sexual stage-specific genes and the knock-out of CDPK1 causes transcriptional dysregulation.

Discussion

Earlier attempts from other groups and our three independent attempts to knock out CDPK1 in the WT *P. falciparum* parasites were not successful (3, 8). The reason for the problem in knocking out *Pf*CDPK1 may be its role in asexual proliferation. In the present study, we demonstrated successful disruption of *Pf*CDPK1 in the CDPK1 T145M parasite background (9), perhaps because of the compensatory mechanisms, mediated through the PKG signaling pathway, for the abnormal CDPK1 T145M. *Pf*CDPK1 is involved in secretion of microneme proteins such as AMA-1 (4) and EBA-175 (7), and this may be the cause for reduced invasion of RBCs in the CDPK1 KO parasites (present study and refs. 4 and 7). In contrast to the CDPK1 KO parasites, the knock-out of *Pb*CDPK1 did not show any asexual growth defect (11), allowing knock-out in the WT parasite background (11).

PKG was shown as a signaling hub for control of egress and invasion in *P. falciparum* (6). The intracellular Ca^{2+} levels were shown to be elevated upon PKG activation in mature schizonts (20) that may be responsible for CDPK5 activation, leading to the egress of merozoites from schizonts (21). Phosphoproteomic analysis revealed *Pf*CDPK1 as a downstream target in the PKG-mediated signaling cascade (6) that likely regulates the invasion of RBCs (20) through phosphorylation of the components of the inner membrane complex (7). The increased sensitivity of CDPK1 KO parasites with C2, an inhibitor of PKG, and an earlier report with CDPK1 T145M parasites (9) highlight the dependence of these parasites on an alternate signaling cascade mediated through PKG. Similarly, PKG was demonstrated in *P. berghei* to mediate release of Ca^{2+} from the internal stores and maintain elevated levels of Ca^{2+}, which acts as an important determinant for the gametocyte activation and subsequent mosquito infection (20).

While our data show the importance of *Pf*CDPK1 in the gametogenesis of *P. falciparum* parasites and transcriptional regulation of sexual stage-specific genes at the asexual stage, *Pb*CDPK1 was shown to be nonessential for gametogenesis (22), although exflagellation was delayed. *Pb*CDPK1 was shown to play a critical role in de-repression of transcripts important for the complete development of ookinetes (22). We looked at the mosquitoes infected with CDPK1 KO for the presence of oocysts, but none occurred. It could be possible that *Pf*CDPK1 also has a role in ookinete development as for *Pb*CDPK1 (22), However, the abnormal presence of the RBC membrane in male and female gametes and the failure of female gametes to round up are the likely cause of the lack of oocysts.

As in the asexual stages, CDPK1 shows peripheral localization in the gametocytes and gametes, suggesting the association of CDPK1 with the inner membrane complex due to the presence of N-terminal motifs that were critical for membrane association (1). Transmission of malaria parasites to mosquitoes is associated with rapid activation of the gametocytes by xanthurenic acid, leading to an increase in intracellular Ca^{2+} concentration that is transformed into male gametocyte exflagellation by CDPK4 (23). Xanthurenic acid is an important factor for exflagellation in vivo as shown in *P. berghei* (24). However, gametogenesis in our studies was induced by reduced temperature and a rise in pH in the absence of xanthurenic acid. CDPK4 was recently demonstrated to play critical roles at various stages of genome replication during the exflagellation process in *P. berghei* (25). In *P. falciparum*, CDPK4 was shown to be a critical kinase during exflagellation using the chemical genetics approach (26), as we have shown for CDPK1 in the present work. The CDPK1 KO parasites were surrounded by the RBC membrane after induction. *Plasmodium* perforin-like protein 2 (PPLP2) is important for the exit of male gametocytes after induction (12, 27). The PPLP2 KO parasites were not able to lyse the RBC membrane after induction, resulting in the formation of a "superflagellum" instead of eight individual flagella (27). The superflagellum was motile, and the PPLP2 KO parasites could establish infection in the mosquitoes, albeit at a much lower rate (27). Clearly, the formation of the superflagellum was a secondary effect of a defect in RBC membrane lysis. In the case of CDPK1 KO, we rarely see the male gametocytes with "superflagellum"-like structure. Moreover, we did not observe movement of the flagellar structures within the RBCs and exflagellation was a rare event, suggesting that the defect is upstream to PPLP2. Taken together, our results and existing information suggest that CDPK1 is critical for male gametocyte exflagellation and that the defect in egress of the male gametes is a secondary effect of its primary role in an upstream signaling pathway.

The exflagellation process was largely unaffected with the CDPK1 KO in *P. berghei*, although the RBC membrane lysis was delayed by 5 min, resulting in flagellar movements while the activated male gametocyte was inside the RBC (22). The male gametes were ultimately released from the RBC. We show that CDPK1 KO in *P. falciparum* is different from its counterpart in *P. berghei* since exflagellation was rarely observed and additionally there was no movement of the flagella inside the RBC and no release of the male gametes even after 20 min of in vitro activation. Importantly, we did not observe oocysts in any of the experiments with the mosquitoes infected with the CDPK1 KO parasites.

Unlike *Pf*CDPK2 KO where female gametes round up and exit the RBC (13), the female gamete formation is defective in *Pf*CDPK1 KO as most do not round up and remain inside the RBC after induction. To the best of our knowledge, there is no report of a kinase that is critical for the female gamete formation in *P. falciparum*.

For insight into the compensatory mechanisms in the CDPK1 KO parasites, we performed global transcriptomics on mature schizonts of WT and CDPK1 KO parasites through RNA-seq. We did not observe a significant up-regulation of a kinase or down-regulation of a phosphatase. This could be due to subtle changes in the transcripts of the kinases and/or phosphatases or posttranscriptional or posttranslational modifications, such as phosphorylation/de-phosphorylation. We observed changes in the transcript expression of members belonging to multigene families such as *P. falciparum* erythrocyte membrane protein 1 and *Plasmodium* helical interspersed subtelomeric family in the CDPK1 KO parasites. Since these changes could result from allelic exclusion between different clones in the parasite population, these genes are not discussed further. Interestingly, we found up-regulation of RKIP. In metazoans, RKIP is a specific inhibitor of Raf-1 in the classical Raf/MEK/ERK, also called the MAPK pathway (28). However, the *Plasmodium* genome does not encode the three classical components of the MAPK pathway (29). Therefore, the function of RKIP remains unknown in *Plasmodium*.

Interestingly, the CDPK1 KO parasites show significant up-regulation of GDV1 and AP2-G. GDV1 is required for gametocyte formation. AP2-G is a master regulator of the transition from the asexual to the sexual phase of the parasite life cycle and was shown to be under a positive feedback regulation (16, 17). These results led us to test the hypothesis that CDPK1 KO

parasites may have increased sexual commitment. However, in the present study, we did not find this to be the case. In plants, CDPKs have been reported to regulate the activity of transcription factors (30, 31). Phosphorylation of a transcription factor by *Nicotiana tabacum* CDPK1 facilitates its sequestration in the cytoplasm (30). Phosphorylation of basic region/leucine-zipper (bZIP) transcription factor by CDPKs is critical for floral transition (vegetative-to-reproductive phase) (31). Our results and studies with plant CDPKs provide sufficient credence to the possibility of transcription factor regulation by *Pf*CDPK1. We also found up-regulation of genes associated with sporozoite biology, such as CelTOS and SIAP1. Taken together, the RNA-seq data suggest programmed developmental dysregulation caused by the knock-out of CDPK1, since there is increased expression of gametocyte markers but no increase in gametocytes per se.

One major concern in work on long-term culture of KO parasites is that other mutations may result in the phenotype seen of reduced asexual development and no infection of mosquitoes. Because of this concern, we complemented the CDPK1 KO by episomal expression of CDPK1. Although complementation partially rescued asexual growth and mosquito infection, it was not identical to the WT. This could be due to overexpression of the CDPK1 WT allele used instead of CDPK1 T145M. However, our complementation data are consistent with another report where complementation of *Pb*MTRAP KO parasites partially rescued (12%) the mosquito infectivity as judged by the presence of oocysts (32). Importantly, successful complementation indicated that the parasites had no unrelated mutations that blocked mosquito infectivity. In our studies on complementation, we compared gametogenesis and mosquito infection between the WT and CDPK1 KO, not with the mutant CDPK1 T145M.

Our study unequivocally demonstrates a critical role of CDPK1 in mosquito infection; and since transmission of the malaria parasite from the human host to mosquitoes is a huge population bottleneck in the parasite life cycle, CDPK1 could be a good target for transmission-blocking drugs. Moreover, a better understanding of compensatory mechanisms in CDPK1 KO parasites may help in devising better strategies for targeting asexual stages of malaria parasites.

Materials and Methods

In Vitro Culture of *P. falciparum*. The NF54 strain of *P. falciparum* was cultured in O+ human RBCs (Virginia Blood Services) under previously defined conditions (9, 33). For the experiments not requiring gametocyte set-up, the parasites were cultivated in 0.05% AlbuMAX II (Thermo Fisher Scientific) instead of 10% heat-inactivated, O+ human sera (Interstate Blood Bank, Inc.). For synchronization of the parasites, see *SI Appendix, Materials and Methods*.

RNA-Seq and Real-Time qPCR. High-quality total RNA was isolated from schizonts (44–48 HPI) of WT and CDPK1 KO parasites using standard procedures. The RNA was used to prepare cDNA libraries (KapaBiosystems) and run on an Illumina HiSeq 3000 instrument to generate 50 million reads per sample. The data were analyzed by edgeR (34). For details, see *SI Appendix, Materials and Methods*.

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