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14. ABSTRACT

Sporozoite infection of the liver is the first obligate step of the *Plasmodium* mammalian cycle. Identifying parasite proteins that are required for liver infection can lead to novel drugs against malaria. For the first time, we report an essential role for two signaling pathways in sporozoite infectivity - cGMP signaling, mediated through the parasite's cGMP-dependent protein kinase (PKG), and Ca^{2+} signaling, mediated through the parasite's calcium-dependent protein kinase 4 (CDPK4). We demonstrated that both enzymes are expressed cytoplasmically in sporozoites and liver stages. Using a specific and potent inhibitor of *Plasmodium* PKG and inhibitor-resistant transgenic parasites, we demonstrated that PKG is essential for sporozoite invasion and consequently infection of hepatocytes. In addition to PKG, CDPK4 is crucial for sporozoite invasion. We showed that inhibiting CDPK4 activity in sporozoites, using either a small molecule inhibitor or conditional deletion of the gene, significantly decreases invasion and infection of hepatocytes. Simultaneous chemical inhibition of PKG and CDPK4 resulted in a cooperative block in sporozoite infection. In conclusion, we have identified two protein kinase signaling pathways that play a key role in sporozoite infection and whose inhibition could be exploited to prevent the first step of a malaria infection. Thus, we have identified two potential targets for development of drugs that restrict liver infection by Plasmodium.

15. SUBJECT TERMS
Plasmodium, sporozoites, liver infection, kinase, drugs

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1. **INTRODUCTION:**
   Sporozoite infection of the liver is the first obligate step of the *Plasmodium* mammalian cycle. Inhibiting this step can block malaria at an early step. However, few anti-malarials target liver infection by sporozoites. Our goal is to find drugs that prevent or control liver infection. Development of such drugs will be facilitated by identification of parasite proteins required for liver infection. These proteins are potential drug targets for development of therapies that restrict Plasmodium liver infection. The aim of this Discovery award is to identify Plasmodium proteins that function in sporozoite invasion of hepatocytes and subsequent intrahepatic development.

2. **KEYWORDS:** Plasmodium, sporozoites, liver infection, kinase, drugs, malaria

3. **OVERALL PROJECT SUMMARY:**

Task 1: Synthesis and chemical characterization of Al-Tsp derivatives begins.

Two classes of Tsp derivatives (Al-Tsp) are appropriate for click chemistry (Fig. 1). Class I derivatives carry a photoaffinity azide group at various positions of the fluoroaryl ring and Class II carry a photoaffinity aryl azide groups in place of the pyridinyl ring. The goal of Task 1 was to synthesize Al-Tsp derivatives.

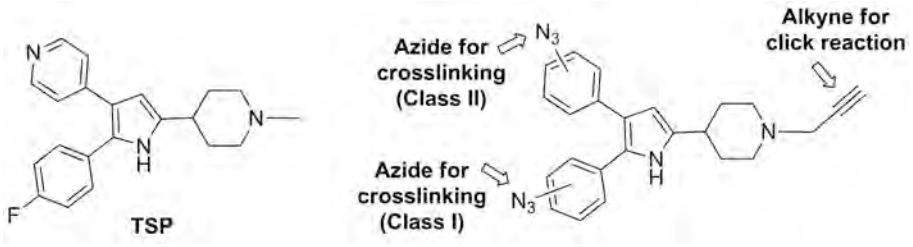

Figure 1: Examples of Tsp derivatives (Al-Tsp). *The crosslinking azide group will be placed at various positions of the fluoroaryl rings in Class I and pyridinyl ring in Class II derivatives.*

Guided by the previously employed methods to synthesize TSP and TSP derivatives, we demonstrated that we could append a propargyl group to the piperidine ring nitrogen and that this derivative retained activity. Thus the propargyl group would serve for click chemistry to isolate TSP derivatives cross-linked to target. Synthesis of aryl azide derivatives of TSP was then pursued, with the expectation to demonstrate activity was retained and that cross-linking could be achieved. Synthetic approaches were pursued to incorporate an azide group into TSP structure with each of the aryl rings. With either aryl ring, addition of an azide group or azide group precursor (nitro or aniline) to TSP or late-stage TSP synthetic intermediate was not achieved. Thus TSP synthetic precursors having a nitro or protected aniline group were prepared, with the expectation that these precursors would be used in the synthesis of the requisite TSP derivatives, and the nitro or aniline groups would be converted to azide at intermediate or final stage of the synthesis. Ultimately, these substitutions to the precursor molecules afforded synthetic intermediates that failed to give desired products when using the methods previously employed to prepare TSP and TSP derivatives. Thus within the limited time-frame and budget of this study, the most direct synthetic routes with fewest synthetic

steps to obtain the target derivatives failed. It is believed that the target azide derivatives are yet synthetically feasible.

However, with failure of the direct synthetic routes using modified intermediates and following the known TSP synthesis to give the target derivatives, future syntheses would require significant time, budget and effort to design and carry out a novel synthesis of TSP derivatives to incorporate the requisite azide groups for crosslinking.

Task 2: Begin HepG2 cell assays with different Al-Tsp derivatives.
Task 3: If AL-TSP identified in Year 1, optimize click chemistry conditions using reactive components. Further optimization carried out using bacterial extracts. If not, continue testing Al-Tsp derivatives. If all molecules are inactive, begin synthesis of Class II Tsp derivatives.

Task 2 and Task 3 were dependent on the successful synthesis of Al-Tsp derivatives in Task 1. Since Task 1 was unsuccessful at the end of year 1, Tasks 2 and 3 could not be undertaken as originally planned. Therefore, we modified our approach so that we could still fulfill the objective of identifying Tsp's target in sporozoites.

To fulfill the project's objective of identifying Tsp's target in sporozoites, we utilized an alternative approach of testing candidate proteins for their role in sporozoite invasion. Using two complementary strategies (a) specific small molecule inhibitors and (b) genetic mutants, we demonstrated that Plasmodium PKG is a target of Tsp during sporozoite invasion.

We used a transgenic *P. berghei* line expressing a HA-tagged, Tsp-resistant allele of PbPKG. The allele carries a substitution of the gatekeeper Thr residue (PKG T_{619}Q-HA) that prevents access of TSP to its binding pocket in P. berghei PKG. *P. berghei* parasites expressing PKG T_{619}Q are significantly less sensitive to inhibition by TSP but undergo normal schizogony, gametogenesis and sporozoite development [1]. We utilized these parasites to examine PbPKG's role in sporozoite infection.

PKG T_{619}Q sporozoites had lower infectivity compared to control sporozoites expressing the wildtype, HA-tagged Tsp-sensitive allele (PKG-HA) (Fig. 2A), suggesting that the PKG plays a role in sporozoite infection. To identify the steps of *Plasmodium's* liver cycle that require PKG, we quantified sporozoite invasion, and intracellular liver stages at different time-points. PKG T_{619}Q-HA sporozoites displayed an approximately two-fold decrease in the fraction of sporozoites that were intracellular 2h after addition to HepG2 cells, suggesting that PKG is required for sporozoite invasion (Fig. 2A). There was no further decrease in the number of intracellular liver stages at 24h post-infection (p.i) and 48h p.i (Fig. 2A). These data suggest that PKG is either not required for or its decreased activity is sufficient for parasite remodeling in the parasitophorous vacuole, nuclear division or intra-vacuolar trophic growth.

To determine if PbPKG is essential for sporozoite infection of hepatocytes, we tested the sensitivity of PKG T_{619}Q-HA sporozoites to Tsp. Infection of HepG2 cells by PKG T_{619}Q-HA sporozoites was about 20-fold less sensitive to Tsp compared to control sporozoites expressing wildtype HA-tagged PKG (PKG-HA) (Fig. 2B). Higher doses of Tsp inhibited infection by both PKG-HA and T_{619}Q-HA sporozoites suggesting that Tsp

could have additional targets secondary to PbPKG (data not shown). Because of these potential off-target effects, higher concentrations of Tsp were not used in subsequent experiments. The refractoriness of T_{619}Q-HA sporozoites to Tsp confirms that sporozoite infection of hepatocytes requires PbPKG, and that PbPKG is the primary target of Tsp in sporozoites. To verify that loss in sporozoite infectivity resulted from a block in sporozoite entry into cells, PKG-HA and PKG T_{619}Q-HA sporozoites were pre-treated with Tsp prior to addition to HepG2 cells. This brief treatment led to a dose-dependent decrease in the number of liver stages only in PKG-HA parasites supporting PbPKG's vital role in sporozoite invasion (Fig. 2B).

Merosome formation and/or release requires PKG. We previously showed that PKG cKO sporozoites do not form merosomes, suggesting that PKG is also required for parasite egress from hepatocytes [2]. These results were confirmed by testing the effect of TSP treatment on merosome formation by PKG-HA or T_{619}Q-HA sporozoites. Addition of TSP to HepG2 cells infected with PKG-HA sporozoites decreased the number of merosomes in the media in a dose-dependent manner (Fig. 2C). In contrast, the number of merosomes in the media of cultures infected with T_{619}Q-HA sporozoites was less sensitive to Tsp treatment. Both genetic and chemical inhibition confirm PKG's essential role in merosome formation and/or release.

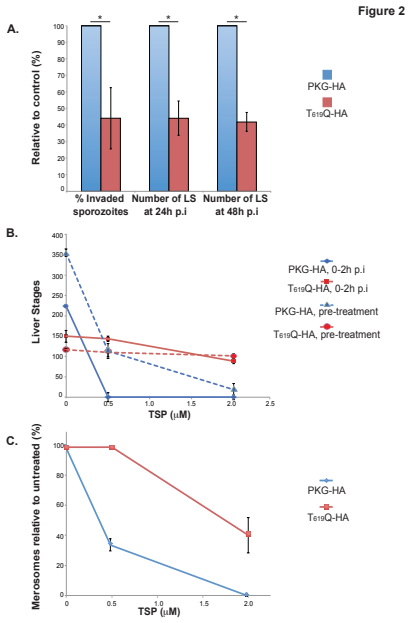

Figure 2: PbPKG is required for sporozoite invasion and merosome formation. *A) Decreased invasion by PKG T_{619}Q sporozoites results in reduced infectivity. PKG T_{619}Q and PKG-HA (control) sporozoites were added to HepG2 cells for 2h to determine the fraction of sporozoites that become intracellular. The number of infected HepG2 cells was determined at 24h and 48h p.i. B) Inhibition of PKG activity blocks sporozoite infectivity. HepG2 cells were infected with PKG T_{619}Q and PKG-HA sporozoites in the presence of TSP for 0-2h p.i (solid lines) or after pre-treatment of PKG T_{619}Q and PKG-HA sporozoites with TSP for 30min (dashed lines). Infected cells were quantified at 44h p.i. C) Inhibition of PKG activity prevents merosome formation. HepG2 cells infected with PKG-HA or T_{619}Q sporozoites were treated with TSP at 24-65h p.i. Number of merosomes released into the media was quantified at 66h p.i. Data was analyzed using two-tailed Mann-Whitney test, * $p < 0.05$.*

These and other results are reported in a manuscript currently have undergone initial review at Molecular Microbiology. The referees found the work to be of interest and made several useful suggestions. The manuscript is being handled as 'major revision'. We are confident that with additional experiments, we will address all major concerns and have the manuscript accepted for publication.

Govindasamy, K., Jebiwott, S., Jaijyan, D.K., Davidow, A. , Ojo, K.K., Van Voorhis, W.C., Brochet, M., Billker, O. and Bhanot, P*. Invasion of hepatocytes by Plasmodium sporozoites requires cGMP-dependent protein kinase and calcium dependent protein kinase 4* (manuscript in review at Molecular Microbiology)

References Cited

1. Brochet M, Collins MO, Smith TK, Thompson E, Sebastian S, et al. (2014) Phosphoinositide metabolism links cGMP-dependent protein kinase G to essential Ca(2)(+) signals at key decision points in the life cycle of malaria parasites. PLoS Biol 12: e1001806.
2. Falae A, Combe A, Amaladoss A, Carvalho T, Menard R, et al. (2010) Role of Plasmodium berghei cGMP-dependent protein kinase in late liver stage development. J Biol Chem 285: 3282-3288.

3. KEY RESEARCH ACCOMPLISHMENTS

- First-ever identification of Plasmodium cGMP dependent protein kinase (PKG) as being key for sporozoite invasion of liver cells.
- Description of Plasmodium cGMP dependent protein kinase's function in sporozoite motility and secretion of micronemal proteins
- First-ever description of Plasmodium calcium dependent protein kinase 4 (CDPK4) as playing an important role in for sporozoite infection of the liver.

4. CONCLUSION:

Our work has implications for therapies aimed at preventing liver infection by *Plasmodium falciparum*. Inhibitors of PKG and CDPK4 with greater potency could significantly decrease the parasite burden in the liver. Another possibility is to use inhibition of PKG and CDPK4 to block multiple parasite stages. Our work together with previous work demonstrates that parasite PKG and CDPK4 are required for both steps of *Plasmodium* transmission – mosquito to mammalian host and mammalian host to mosquito. Our work on sporozoite infection demonstrates the role of PKG and CDPK4 during transmission of parasites from mosquito to mammalian host. Previous reports demonstrate that PKG and CDPK4 are required for parasite development in the mosquito midgut. Since both PKG and CDPK4 can be inhibited with small molecule inhibitors that are selective over most host protein kinases, they could be attractive targets for both prophylaxis and transmission blocking approaches.

Future work will focus on synthesis and testing of small molecule inhibitors of PKG and CDPK4 with the aim of discovering novel potent inhibitors of both steps Plasmodium transmission.

5. PUBLICATIONS, ABSTRACTS, AND PRESENTATIONS:

a. List all manuscripts submitted for publication during the period covered by this report resulting from this project. Include those in the categories of lay press, peer-reviewed scientific journals, invited articles, and abstracts. Each entry shall include the author(s), article title, journal name, book title, editors(s), publisher, volume number, page number(s), date, DOI, PMID, and/or ISBN.

(1) Lay Press: None
(2) Peer-Reviewed Scientific Journals:
Govindasamy, K., Jebiwott, S., Jaijyan, D.K., Davidow, A. , Ojo, K.K., Van Voorhis, W.C., Brochet, M., Billker, O. and Bhanot, P*. Invasion of hepatocytes by Plasmodium sporozoites requires cGMP-dependent protein kinase and calcium dependent protein kinase 4* (manuscript in review at Molecular Microbiology)
(3) Invited Articles: None
(4) Abstracts:
Bhanot, P., Govindasamy, K., Khan, R. , Ojo, K.K., Van Voorhis, W.C., Brochet, M., Billker, O., Turk, B. and Bhanot, P. *'Invasion of hepatocytes by Plasmodium sporozoites requires cGMP-dependent protein kinase and calcium dependent protein kinase 4'* (2015) at the Molecular Parasitology Meeting XXVI

b. List presentations made during the last year (international, national, local societies, military meetings, etc.). Use an asterisk (*) if presentation produced a manuscript.
Oral presentation at the Molecular Parasitology Meeting XXVI, 2015

6. **INVENTIONS, PATENTS AND LICENSES:**
None

7. **REPORTABLE OUTCOMES:**
Journal publications (submitted): Govindasamy, K., Jebiwott, S., Jaijyan, D.K., Davidow, A. , Ojo, K.K., Van Voorhis, W.C., Brochet, M., Billker, O. and **Bhanot, P.** *Invasion of hepatocytes by Plasmodium sporozoites requires cGMP-dependent protein kinase and calcium dependent protein kinase 4*

8. **OTHER ACHIEVEMENTS:**
As part of this grant, we developed a novel parasite line that lacks CDPK4 protein in sporozoites (CDPK4 cKO). This line will be available to the community pending publication.

9. **REFERENCES:**
None

10. **APPENDICES:**
Attached is a copy of the manuscript that underwent review at Molecular Microbiology. It was judged to be of interest and is being handled as 'Major

revision’. We are currently in the process of addressing reviewer concerns prior to resubmission.

Invasion of hepatocytes by *Plasmodium* sporozoites requires cGMP-dependent protein kinase and calcium dependent protein kinase 4

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Summary

Successful invasion of hepatocytes by sporozoites is essential for *Plasmodium* to initiate infection of the mammalian host. The parasite's subsequent intracellular development in the liver is the first developmental step of its mammalian cycle. Despite their clinical and biological significance, surprisingly little is known of the signaling pathways required for sporozoite invasion. We report that sporozoite invasion of hepatocytes requires signaling through two second-messengers - cGMP mediated by the parasite's cGMP-dependent protein kinase (PKG), and Ca^{2+}, mediated by the parasite's calcium-dependent protein kinase 4 (CDPK4). Sporozoites expressing a mutated form of *P. berghei* PKG or carrying a deletion of the CDPK4 gene were defective in invasion of hepatocytes. Using specific and potent inhibitors of *Plasmodium* PKG and CDPK4, we demonstrated that PKG and CDPK4 are required for sporozoite motility. Chemical inhibition of PKG decreased parasite egress from hepatocytes by inhibiting either the formation or release of merosomes. In contrast, genetic or chemical inhibition of CDPK4 in sporozoites does not significantly decrease parasite egress from infected hepatocytes. By revealing the requirement for PKG and CDPK4 in *Plasmodium* sporozoite invasion, our work adds to a comprehensive understanding of kinase pathways that act in different *Plasmodium* stages.

Abbreviated Summary

Malaria infection begins with the injection of *Plasmodium* sporozoites into humans by a feeding mosquito. We demonstrate that two parasite kinases, PKG and CDPK4 are crucial for sporozoite motility and consequently their entry into hepatocytes. Our results have implications for understanding the role of second messenger molecules, cGMP and Ca^{2+} in regulating the exo-erythrocytic cycle of *Plasmodium*.

Introduction

Malaria infection begins with the infection by *Plasmodium* 'sporozoites' of the liver. After asexual replication in the liver to form 'liver stages', the parasites invade erythrocytes and replicate to produce merozoites and gametocytes. Merozoites initiate repeated rounds of erythrocytic invasion and asexual development that cause disease. Gametocytes initiate the sexual cycle in the mosquito that is essential for continued parasite transmission. Sexual development in the mosquito leads to stages known as ookinetes. Ookinetes undergo differentiation in the mosquito midgut to form sporozoites. Sporozoites are carried from the mosquito midgut to the salivary glands, from where they can commence another round of parasite transmission to the mammalian host.

The first obligate step of malaria in the mammalian cycle is the infection of hepatocytes by *Plasmodium* sporozoites. The increase in parasite numbers at this stage is essential for the parasite to establish a niche in the mammalian host (Graewe *et al.*, 2012). Decreasing sporozoite infection of the liver decreases both the incidence and severity of disease (Alonso *et al.*, 2005). A mechanistic understanding of sporozoite invasion could reveal pathways that may be targeted for preventing malaria.

In addition to its clinical relevance, invasion by *Plasmodium* sporozoites is biologically significant because it displays a unique combination of features (Meissner *et al.*, 2013). Sporozoites are motile over a large distance as they leave the site of inoculation in the skin to reach the liver. During this process, they migrate through several cells, breaching the host cell plasma membrane in the process. Once an appropriate host hepatocyte is encountered, they switch their mode of cell entry to one accompanied by the formation of a vacuole that serves as the site for further development (Coppi *et al.*, 2007) (Risco-Castillo *et al.*, 2015). While *Plasmodium* ookinetes, like sporozoites, are motile and migrate through the mosquito midgut epithelium, their invasion does not involve the formation of a parasitophorous vacuole. While *Plasmodium* merozoites, like sporozoites, invade forming a parasitophorous vacuole, they are not motile and do not migrate through cells since they are released in close proximity to their target cells. Therefore, *Plasmodium* sporozoites are an excellent model for studying the complexity of Apicomplexan invasion.

Sporozoite invasion of hepatocytes is triggered through a cascade of signaling events initiated by interaction between the circumsporozoite protein on the sporozoite surface and the highly negatively charged heparan sulfate proteoglycans on the hepatocyte surface (Coppi *et al.*, 2007). These signaling events regulate diverse processes in the sporozoite, such as protein secretion from specialized organelles, Ca^{2+} mediated signaling and processing of surface adhesins (Ejigiri & Sinnis, 2009). How these diverse pathways are regulated in sporozoites is unknown. Evidence from other life cycle stages of *P. falciparum* and *P. berghei* has shown the parasite's cyclic GMP dependent protein kinase (PKG) plays an essential role as an upstream regulator of Ca^{2+} signals during both the mammalian and the mosquito cycle (Brochet *et al.*, 2014). During the mammalian cycle, PKG is required for merosome formation and/or release in the liver (Falae *et al.*, 2010), erythrocytic stage schizogony, merozoite invasion and egress

(Collins *et al.*, 2013, Taylor *et al.*, 2010). During the mosquito cycle, PKG is required for gametogenesis and ookinete motility (McRobert *et al.*, 2008, Moon *et al.*, 2009). Here we study the role of PKG in *P. berghei* sporozoite biology.

We show that *P. berghei* PKG (PbPKG) is a key regulator of sporozoite motility that is a pre-requisite for sporozoite invasion of hepatocytes. In addition, *P. berghei* calcium dependent protein kinase 4 (PbCDPK4) contributes to sporozoite motility and invasion. Indirectly our data implicate cGMP and Ca^{2+} as important second messengers for regulating sporozoite invasion. In addition, we demonstrate that PbPKG but not PbCDPK4 is required for the formation and/or release of merosomes that allow parasites to exit the infected hepatocyte. Our results have implications for understanding the network of kinase interactions at different parasite stages and for therapies aimed at multiple parasite stages.

Results

Hepatocyte invasion by sporozoites requires PbPKG. To determine if PKG is expressed in sporozoites, we examined expression of HA-tagged *P. berghei* PKG (PbPKG) under the control of its endogenous promoter using immunofluorescence assays (IFA) (Fig 1A, Supplementary Fig 1A [8]). We find PbPKG distributed throughout the cytoplasm of sporozoites and in liver stages, suggesting a functional role for PbPKG in these stages. A function for PKG in these stages is further supported by the ability of a selective inhibitor of Apicomplexan PKG, a trisubstituted pyrolle (TSP) known as Compound 1 (Gurnett *et al.*, 2002) to potently block sporozoite infection *in vitro* and *in vivo* (Panchal & Bhanot, 2010).

TSP's efficacy against sporozoites would suggest that its primary target, PKG is required for sporozoite infection of hepatocytes. However, this is at odds with our previous genetic data demonstrating that salivary gland sporozoites generated using excision of the PbPKG open reading frame in developing midgut sporozoites (PbPKG cKO) did not display a significant decrease in sporozoite infection (Falae *et al.*, 2010). The failure of stage-specific PbPKG gene excision to reveal a phenotype in sporozoite infectivity could be explained by the carryover of PbPKG protein from oocysts, which contain an intact PbPKG locus, into sporozoites that develop from them. Carryover of PbPKG protein is highly likely since stable isotope labeling of ookinete cultures has demonstrated that 87% of PbPKG protein in ookinetes is inherited from the preceding gamete stages, suggesting that the protein can turnover very slowly (Sebastian *et al.*, 2012).

Using antisera raised against a carboxy terminal peptide of PbPKG, we readily detected PKG protein in PbPKG cKO sporozoites by IFA (Fig 1B). In contrast, PKG protein expression in PbPKG cKO liver stages was significantly reduced. These results support our hypothesis that cKO sporozoites retain sufficient PbPKG protein. They suggest that PKG function in sporozoites is best examined using the available fast-acting and specific chemical inhibitors. Indeed this approach was useful for functional ablation of PKG orthologs in *T. gondii* (Donald *et al.*, 2002) and in *Plasmodium* schizonts and

sexual stages (Brochet *et al.*, 2014, McRobert *et al.*, 2008, Taylor *et al.*, 2010).

To rule out off-target effects of TSP on hepatocyte invasion, we generated a transgenic *P. berghei* line expressing a 3xHA-tagged, TSP-resistant allele of PbPKG using a strategy validated previously (Brochet *et al.*, 2014) but with a GFP-expressing reference line to facilitate monitoring of parasite movement and development (Supplementary Fig 1A-C). The modified PbPKG allele carries a substitution of the 'gatekeeper' residue, Thr_{619} (PKG T_{619}Q-HA) that prevents TSP from accessing its binding pocket. Therefore, PKG T_{619}Q is resistant to TSP while maintaining normal catalytic efficiency (Donald *et al.*, 2002). As a control, we used the line expressing 3xHA-tagged wildtype, inhibitor-sensitive PbPKG (PKG-HA) that was integrated in an identical manner to the PKG T_{619}Q-HA allele (Supplementary Fig 1A-C). Asexual stage parasites expressing PKG T_{619}Q-HA contain normal amounts of PbPKG protein 9 Supplementary Fig 1D), and undergo grossly normal asexual and sexual development (Supplementary Fig 1E, (Brochet *et al.*, 2014)). However, calcium mobilization in gametocytes and ookinetes expressing PKG T_{619}Q-HA, as well as motility in ookinetes expressing PKG T_{619}Q-HA, are significantly less sensitive to inhibition by TSP (Brochet *et al.*, 2014).

To determine if PbPKG is essential for sporozoite infection of hepatocytes, we tested the sensitivity of PKG T_{619}Q-HA sporozoites to TSP. Sporozoites were allowed to infect HepG2 cells in the presence of TSP and compound exposure was maintained for 14 h post infection (p.i.) before the number of infected cells was quantified at 44 h p.i.. Infection of HepG2 cells by PKG T_{619}Q-HA sporozoites was at least 4-fold less sensitive to TSP compared to infection by PKG-HA sporozoites (Fig. 2A, Supplementary Table 1A). While treatment with 0.5 μM TSP eliminated infection by PKG-HA sporozoites, it did not significantly decrease infection by PKG T_{619}Q-HA sporozoites. In fact, even 10 μM TSP did not abolish infection by PKG T_{619}Q-HA sporozoites (Supplementary Table 1A). The refractoriness of PKG T_{619}Q-HA sporozoites to TSP demonstrates clearly that TSP inhibits sporozoite infection by acting on PKG and that sporozoite infection of hepatocytes requires PKG.

We previously observed that TSP did not decrease the number of liver stages when added 3 h p.i. (Panchal & Bhanot, 2010), suggesting that PKG's critical functions are during early steps of sporozoite infection. To further investigate the steps at which PKG is important, PKG-HA and PKG T_{619}Q-HA sporozoites were pre-treated with TSP for 30 min prior to infection. Upon subsequent addition to HepG2 cells, compound was diluted to levels ineffective when tested alone. At 2 h p.i., existing media was replaced with compound-free media. The exposure of sporozoites to TSP prior to addition to HepG2 cells was sufficient to significantly decrease the number of liver stages formed by PKG-HA but not PKG T_{619}Q sporozoites at 44 h p.i. (Fig 2A, Supplementary Table 1A). These results raise the possibility that PbPKG's major role in sporozoite infection is around the point of host-cell invasion rather than subsequent trophic growth.

Sporozoite motility requires PKG. Sporozoite invasion consists of three loosely defined steps – attachment to the substrate, motility to reach the target cells and entry into the host cell (Meissner *et al.*, 2013). PKG is required for secretion of micronemal

adhesins and motility in *T. gondii* tachyzoites (Wiersma *et al.*, 2004) and for motility in *Plasmodium* ookinetes (Moon *et al.*, 2009). By analogy to its roles in these zoites, we hypothesized that PKG's role around the time of sporozoite invasion reflects its function in sporozoite motility.

We tested PKG's role in sporozoite motility by filming PKG-HA and PKG T_{619}Q-HA sporozoites for 120sec at 1Hz. *S*porozoite movement patterns *in vitro* were categorized as previously described (Hegge *et al.*, 2009) - (i) 'gliding' which describes sporozoites moving in circular tracks for the entire observation period of 120sec (ii) 'adherent' which describes sporozoites adhering to the substrate with minor displacement (iii) 'waving' which describes sporozoites attached to the substrate at one end with the other end moving freely in the media termed (iv) 'complex' which describes sporozoites that display a combination of these simple patterns, for example those that glide for part of the time, detach and move out of the field of observation. Sporozoites that attached weakly to the substrate moving in the direction of media flow were categorized as 'drifting'.

Motility of PKG-HA, but not PKG T_{619}Q-HA, sporozoites was highly sensitive to TSP. Treatment with 0.5 μM TSP significantly decreased the percentage of PKG-HA sporozoites that glide (Fig 2B, Supplementary Fig 2, Supplementary Table 1B) and the number of circles executed during the observation period by the gliding sporozoites (from an average of 15.7±1.7 circles/sporozoite to 12.7±1.5 circles/sporozoite in 180 seconds). The number of circles made by sporozoites in unit time is an accurate proxy for their speed of movement (Hegge *et al.*, 2010). In addition, in the absence of TSP, a smaller percentage (± standard error, n = 2 experiments) of PKG T_{619}Q-HA sporozoites glide compared to PKG-HA sporozoites: 6.45 ± 0.99% versus 19.1 ± 1.65%, respectively (Supplementary Table 1B).

To rule out the possibility that HA-tagged PKG may have subtle functional differences from untagged PKG, we also examined motility in PbGFP-Luc sporozoites (Franke-Fayard *et al.*, 2005), which contain an unmodified PKG locus. We found that PbGFP-Luc sporozoites were also robustly inhibited by TSP, although they were less sensitive to the lower doses of TSP compared to PKG-HA sporozoites (Supplementary Table 1C). This difference may be due to different genetic backgrounds, or could reflect subtle effects of the HA-tag and generic 3' UTR on PKG-HA, leading to the sensitivation of PKG-HA to TSP. At 2 μM, TSP reduced not only the percentage of gliding sporozoites but also the number of circles they make - 13.7±4.7 circles/sporozoite for vehicle-treated to 7.0±1.7 for 2.0 μM-treated sporozoites (Supplementary Table 1B).

We noted that in addition to decreased motility, TSP-treated PbGFP-Luc sporozoites were unable to attach strongly to the substrate and showed a linear displacement of 25-50 μm in the direction of medium flow (Supplementary Table 1B, Supplementary Fig. 2). These sporozoites were characterized as 'drifting'. Weak initial attachment to the substrate would severely impair motility. Therefore, PKG could also be required for subsequent cycles of attachment-detachment from the substrate that occur during

translocation or for generating the force needed for movement (Hegge *et al.*, 2010, Munter *et al.*, 2009).

Together, these data demonstrate PKG's key role in sporozoite motility and suggest that inhibition of PKG may also decrease sporozoite adhesion to the surface. Since motility is required for invasion, PKG most likely regulates sporozoite invasion by controlling motility.

Merosome formation and/or release requires PKG. We previously showed that PKG cKO sporozoites do not form merosomes, suggesting that PKG is required for parasite egress from hepatocytes (Falae *et al.*, 2010). These results were confirmed by testing TSP's effect on merosome formation by PKG-HA or PKG T_{619}Q-HA parasites. Addition of TSP to HepG2 cells infected with PKG-HA sporozoites decreased the number of merosomes found in the media at 65 h p.i., in a dose-dependent manner (Fig 2C, Supplementary Table 1A). In contrast, merosome formation and/or release by PKG T_{619}Q-HA sporozoites was less sensitive to TSP treatment. Therefore, genetic and chemical inhibition confirm PKG's essential role in merosome formation and/or release.

The T619Q mutation has subtle effects in the absence of inhibitor. Despite undergoing normal intraerythrocytic development (Supplementary Fig 1E, (Brochet *et al.*, 2014)) and sexual development *in vitro* (Brochet *et al.*, 2014), PKG T_{619}Q-HA parasites consistently produced only about half as many liver stages as the isogenic PKG-HA control clone (Supplementary Fig 1F, Supplementary Table 1C). This loss of infectivity seemed to happen at the point of invasion since we observed that PKG T_{619}Q-HA parasites displayed an approximately two-fold decrease in the fraction of sporozoites that were intracellular 2 h after addition to HepG2 cells (Supplementary Fig 1F, Supplementary Table 1C). Following invasion, the T_{619}Q mutation did not further impact the number of intracellular liver stages that develop at 24 h p.i. and 48 h p.i. (Supplementary Fig. 1F, Supplementary Table 1C). These differences could be a result of unknown genetic differences amongst the nominally isogenic PKG T_{619}Q-HA and PKG-HA clones, although we find it more likely that PKG T_{619}Q-HA is a hypomorphic allele in sporozoites.

We attempted to compare PKG protein levels amongst PKG T_{619}Q-HA and PKG-HA sporozoites by Western blotting but were unable to collect sufficient material. As an alternative, we quantified anti-HA immunofluorescence intensities with anti-GFP as an internal reference control in immunofluorescence assays. The average ratio of anti-HA to anti-GFP fluorescence intensity in PKG T_{619}Q-HA sporozoites was 0.17 $\pm$ 0.04 (n = 78 sporozoites) and in PKG-HA sporozoites was 0.24 $\pm$ 0.04 (n = 70 sporozoites). The difference in the sporozoite populations was statistically significant (p value = 7.73E-07, unpaired t-test of log transformed ratios, Supplementary Fig 1G), and similar results were obtained in three independent experiments. In contrast, levels of PKG protein in erythrocytic stage parasites from the two lines were very similar (Supplementary Fig 1D), consistent with the normal erythrocytic cycle of the PKG T_{619}Q-HA parasites (Supplementary Fig 1E). We conclude that decreased PKG expression in PKG T_{619}Q-HA sporozoites is a possible cause of their reduced infectivity. In addition, PKG T_{619}Q-

HA enzyme could have reduced activity *in vivo* as seen in 'gatekeeper' mutants of some kinases (Zhang *et al.*, 2005). Since PKG T_{619}Q-HA sporozoites have significantly decreased infectivity whereas PbPKG cKO sporozoites do not (Falae *et al.*, 2010), we hypothesize that PKG enzymatic activity is closer to wildtype levels in PbPKG cKO sporozoites because of the presence of a significant amount of PKG protein in the cKO sporozoites. (Fig. 1B)

PbCDPK4 plays a crucial role in sporozoite invasion of hepatocytes. PKG-dependent pathways include phosphoinositide metabolism, protein secretion, vesicular trafficking, proteolysis, gene regulation and cellular signaling (Alam *et al.*, 2015, Brochet *et al.*, 2014). Many of PKG's pleiotropic roles in different parasite stages are likely explained by its regulation of critical Ca^{2+} signals that control merozoite invasion and egress, gametocyte activation and ookinete motility (Alam *et al.*, 2015, Brochet *et al.*, 2014). In a model developed in *P. berghei* ookinetes, gametocytes and *P. falciparum* schizonts, PKG-dependent phosphatidylinositol (4,5)-biphosphate production releases internal Ca^{2+} in the parasite (Brochet *et al.*, 2014). The resulting Ca^{2+} flux is transduced by Ca^{2+} effectors, including a family of calcium dependent protein kinases (CDPK). Different CDPKs act downstream of PKG at various steps of the parasite life-cycle – CDPK1 during merozoite invasion (Alam *et al.*, 2015), CDPK5 during merozoite egress (Dvorin *et al.*, 2010), CDPK3 during ookinete but not sporozoite motility (Siden-Kiamos *et al.*, 2006) and CDPK4 in microgametogenesis (Billker *et al.*, 2004). The specific CDPK that acts downstream of PKG in sporozoite and liver stages has not been identified, although efficient invasion of hepatocytes requires CDPK6 (Coppi *et al.*, 2007).

Elevated Ca^{2+} levels in gliding sporozoites suggest that sporozoite motility requires Ca^{2+} signaling pathways (Carey *et al.*, 2014). However, the identity of these pathways is as yet unknown. Since CDPKs are major mediators of Ca^{2+} signaling in *Plasmodium* and distinct CDPKs act downstream of PKG throughout the parasite life-cycle (Billker *et al.*, 2004, Dvorin *et al.*, 2010, Sebastian *et al.*, 2012, Siden-Kiamos *et al.*, 2006), we hypothesized that sporozoite motility and invasion is likely to require CDPK activity. We focused on CDPK4 whose function in sporozoites has not yet been examined. Using antisera against *T. gondii* CDPK1 which cross-reacts with *P. berghei* CDPK4 (Billker *et al.*, 2004), we determined that CDPK4 is present in sporozoites (Fig. 3A). Since *P. berghei* CDPK4 (PbCDPK4) is essential for male gametogenesis, CDPK4 knockout parasites do not infect mosquitoes and consequently, cannot produce sporozoites (Billker *et al.*, 2004). Therefore, we generated a stage-specific knockout (cKO) allele using the FlpL/FRT system (Lacroix *et al.*, 2011) (Fig. 3B). In the CDPK4 cKO line, the CDPK4 open reading frame is excised during development in the mosquito midgut, generating a sporozoite population in which CDPK4 expression was below the level of detection (Fig. 3A). CDPK4 expression in liver stages of CDPK4 cKO line was similarly significantly reduced (Fig. 3A). Immunofluorescence assays were utilized since sufficient numbers of sporozoites for Western blot analysis could not be obtained. TRAP/FlpL parasites (Panchal *et al.*, 2012), the FlpL-expressing parent line used to modify the CDPK4 locus, served as controls. CDPK4 is not required for sporozoite formation since CDPK4 cKO sporozoites developed normally (8175$\pm$1247

sporozoites/mosquito for TRAP/FlpL-infected mosquitoes and 6887±765 sporozoites/mosquito for CDPK4 cKO-infected mosquitoes). These sporozoites were used to assess the role of CDPK4 in sporozoite infection and the liver cycle.

Hepatocyte invasion by CDPK4 cKO sporozoites was examined by quantifying the fraction of sporozoites that are intracellular 2 h after addition to host cells. In CDPK4 cKO infected cells, there was a two-fold decrease in the fraction of intracellular sporozoites compared to control sporozoites (Fig 4A, Supplementary Table 2A), suggesting an important role for CDPK4 in sporozoite entry into host cells. To determine if CDPK4 has additional functions during intrahepatic development, we quantified the number of liver stages present in infected HepG2 cultures at 24 h p.i. and 48 h p.i.. CDPK4 cKO sporozoites form half as many liver stages compared to control sporozoites (Fig 4A, Supplementary Table 2A), a decrease equivalent to the reduction in sporozoites that invade hepatocytes. These data suggest that PbCDPK4 does not have an additional role in intracellular development after the sporozoite has successfully invaded the hepatocyte.

A potential role for CDPK4 during egress from hepatocytes was examined by quantifying merosome formation in cKO and control cultures. To compensate for the two-fold lower infectivity of CDPK4 cKO sporozoites and ensure similar numbers of liver stages leading up to merosome formation and/release, we infected HepG2 cells with twice as many cKO sporozoites compared to control. The numbers of intracellular liver stages and merosomes in the media were quantified at 65-72 h p.i. CDPK4 cKO sporozoites were not significantly affected in their ability to form merosomes (Fig 4A, Supplementary Table 2A). Our data suggest that CDPK4's most important role in pre-erythrocytic stages is during sporozoite invasion of hepatocytes and that it does not have a major role in parasite egress from hepatocytes.

CDPK4's effect on parasite infection in mice was examined by determining the pre-patent period in mice (days to appearance of erythrocytic stage parasites in Giemsa-stained blood smears) following intravenous injection of $1x10^4$ sporozoites/mouse. In the first experiment, deletion of CDPK4 increased the pre-patent period from 4.2 ± 0.22 days (n=5, 5/5 control-infected mice developed blood stage parasitemia) to 4.75 ± 0.25 days (n=4, 4/4 CDPK4 cKO-infected mice developed blood stage parasitemia). Average parasitemias at days 4 and 6 post-infection were not significantly different in the two groups (Supplementary Table 2B). In a repeat experiment, the pre-patent periods of control sporozoites was 4.0 ± 0.46 days (n=10, 8/10 mice control-infected mice developed blood stage parasitemia) and of cKO sporozoites was 4.66 ± 0.25 days (n=10, 6/10 CDPK4 cKO-infected mice developed blood stage parasitemia). While at day 4 in the second experiment, blood parasitemias of the two groups were significantly different (p value<0.05, unpaired t-test), there was only a trend towards delay in patency of CDPK4 cKO sporozoites that did not reach significance (Supplementary Table 2B). The effect size in both replicates is however, entirely consistent with the two-fold decrease in hepatocyte infection by cKO sporozoites observed *in vitro* since a 1-day delay in patency determined by microscopic examination of blood smears would require a 10-fold decrease in liver parasitemia.

Next we examined if CDPK4's role in invasion could result from its function in sporozoite motility. We utilized a bumped kinase inhibitor compound 1294 that is relatively specific for CDPK4 *in vitro* since it exploits the small 'gatekeeper' residue of the kinase that is absent in most *Plasmodium* kinases (Ojo *et al.*, 2014, Ojo *et al.*, 2012). In *P. falciparum*, treatment of gametocytes with 1294 phenocopies the effects of deleting CDPK4 by inhibiting male gametogenesis, thus blocking oocyst development. Importantly, inhibition of oocyst development by 1294 was reversed by introducing a larger amino acid in the 'gatekeeper' position of *P. falciparum* CDPK4, demonstrating that CDPK4 is the primary *in vivo* target of 1294 (Ojo *et al.*, 2014, Ojo *et al.*, 2012). Although PKG also has a small 'gatekeeper' residue, the IC_{50} of 1294 against PfCDPK4 (10 nM) is a log lower than against PfPKG (200 nM) (Ojo *et al.*, 2014, Ojo *et al.*, 2012).

Using the same gliding assay as before, we found that 0.5 μM 1294 significantly decreased the percentage (± standard error, n = 2 experiments) of sporozoites that glide from 29.67 ± 1.91% to 17.29 ± 1.4% (Fig 4B, Supplementary Table 2C). These data indicate that CDPK4 may be one of a number of effectors that mediate the Ca^{2+} fluxes observed in gliding sporozoites, and which are associated with micronemal secretion and motility (Carey *et al.*, 2014).

Discussion

Our work adds to a comprehensive understanding of the role of cGMP and Ca^{2+} signaling in apicomplexans. It extends the role of parasite cGMP and Ca^{2+} signaling pathways, as mediated by PKG and CDPK4, to sporozoites in addition to their previously recognized roles in asexual and liver stages (PKG) as well as sexual stages (PKG and CDPK4). We clearly demonstrate an essential role for PKG and an important role for CDPK4 in invasion of hepatocytes by *Plasmodium* sporozoites that has not been previously appreciated (Fig. 2A and Fig. 4A). The function of the two enzymes in sporozoite invasion may well be explained by their roles in sporozoite motility (Fig. 2B, Fig. 4B). While PKG is additionally required for the formation of merosomes through which parasites exit the hepatocyte (Fig. 2C, (Falae *et al.*, 2010)), the same knockout strategy has not produced evidence linking CDPK4 to liver stage egress (Fig. 4A). It is possible that CDPK4 has a subtle role in merosome formation and release, which is not detected in the current assay.

PKG is known to regulate micronemal secretion in *P. falciparum* merozoites (Collins *et al.*, 2013) and in *T. gondiii* tachyzoites (Wiersma *et al.*, 2004). By analogy, we hypothesize that PKG's regulation of sporozoite motility occurs through regulation of exocytosis of micronemal adhesins onto the sporozoite surface. Future work will have to elucidate whether PKG regulates the release of known sporozoite adhesins such as TRAP, TRAP-like protein and S6 (Sultan *et al.*, 1997, Heiss *et al.*, 2008, Moreira *et al.*, 2008, Combe *et al.*, 2009, Steinbuechel & Matuschewski, 2009, Hegge *et al.*, 2010). The exact contribution of each adhesin to the formation of the primary attachment site between the sporozoite and the substrate, turnover of adhesion sites and the generation of the force needed to propel sporozoites remains to be established using reflection interference contrast, traction force and/or total internal reflection fluorescence

436 microscopy (Hegge *et al.*, 2010). By regulating protein secretion in sporozoites, PKG
437 could also regulate the formation of the 'tight junction' between the zoite and the host
438 cell that serves as an anchor point during sporozoite entry into hepatocytes (Giovannini
439 *et al.*, 2011). The 'tight junction' is thought to require the release of micronemal proteins,
440 some of which may be regulated by PKG activity. In this way, PKG could contribute to
441 sporozoite invasion both through the regulation of motility and 'tight junction' formation.
442
443 Our results suggest that a previously published model for microgamete activation in
444 which PKG acts upstream of CDPK4 to transduce effects of Ca^{2+} released through PKG
445 signaling (Brochet *et al.*, 2014) likely extends to sporozoite motility. However, since
446 CDPK4 inhibition produces a relatively less severe phenotype compared to PKG
447 inhibition, we propose that additional Ca^{2+} mediators are involved. We recognize that
448 low amounts of functional CDPK4 protein in cKO sporozoites, that would have to be
449 below the level of detection by IFA, cannot be ruled out.
450
451 Together with previous work (Dvorin *et al.*, 2010, McRobert *et al.*, 2008, Moon *et al.*,
452 2009, Taylor *et al.*, 2010, Wiersma *et al.*, 2004) these studies provide an opportunity to
453 trace the evolution of orthologous parasite kinases across parasite stages and species.
454 This is the first time we identify a stage-transcending requirement for a *Plasmodium*
455 CDPK. Our data suggest that CDPK4 has an important but likely non-essential role in
456 sporozoite invasion (although the possibility of residual protein that would have to be
457 below the level of detection by IFA cannot be ruled out). This is in contrast to the same
458 enzyme's essential role in male gametogenesis (Billker *et al.*, 2004) or to CDPK5's
459 essential role in merozoite egress (Dvorin *et al.*, 2010). PbCDPK4 shares with its *T.*
460 *gondii* ortholog, TgCDPK1, a role in regulating zoite gliding and invasion, which in *T.*
461 *gondii* was shown to be due to its requirement for microneme secretion (Lourido *et al.*,
462 2010). However, unlike TgCDPK1 (Lourido *et al.*, 2012), PbCDPK4 does not have a
463 major role in parasite egress at any life cycle stage.
464
465 We were intrigued to find that PbPKG T_{619}Q-HA sporozoites have a phenotypic effect in
466 the parasite that has not been previously observed in erythrocytic stages and ookinetes
467 carrying the same mutation (Brochet *et al.*, 2014) (Fig. 2F). Similarly, the equivalent
468 substitutions in PKG in other species did not impact gametogenesis and schizogony of
469 *P. falciparum* (McRobert *et al.*, 2008, Taylor *et al.*, 2010) or growth of *T. gondii*
470 tachyzoites (Donald *et al.*, 2002). Compared to PbPKG T_{619}Q-HA, PbPKG cKO
471 sporozoites have normal infectivity because they contain wildtype enzyme. We propose
472 that the T_{619}Q substitution causes subtle differences in enzyme stability *in vivo* as is
473 reported for 'gatekeeper' mutations in several other kinases, including PfPKG
474 (McRobert *et al.*, 2008, Zhang *et al.*, 2005). *P. falciparum* PKG in which the gatekeeper
475 Thr is substituted by Met is unexpectedly more sensitive to TSP implying a subtle
476 change in enzymatic function (McRobert *et al.*, 2008).The sporozoite-specific effect of
477 the T_{619}Q mutation raises the intriguing possibility that PKG has stage-specific
478 interactions that affect its stability in sporozoites and have functional consequences in
479 the form of decreased sporozoite motility and invasion.
480
481 Our work has implications for therapies aimed at preventing liver infection by

Plasmodium. Inhibitors of PKG and CDPK4 with greater potency could significantly decrease the parasite burden in the liver. Another possibility is to use inhibition of PKG and CDPK4 to block multiple parasite stages. Work presented here together with previous reports [8,23] demonstrate that parasite PKG and CDPK4 are required for both steps of *Plasmodium* transmission – mosquito to mammalian host and mammalian host to mosquito. The current work on sporozoite infection demonstrates the role of PKG and CDPK4 during transmission of parasites from mosquito to mammalian host. Previous reports demonstrate that PKG is required for male and female gametogenesis (McRobert *et al.*, 2008) and CDPK4 is required for male specific gametogenesis events in the mosquito (Billker *et al.*, 2004). Since both PKG and CDPK4 can be inhibited with small molecule inhibitors that are selective over most host protein kinases (Brochet *et al.*, 2014, Collins *et al.*, 2013, Ojo *et al.*, 2014, Vidadala *et al.*, 2014), they could be attractive targets for both prophylaxis and transmission blocking approaches.

Experimental Procedures

Ethics Statement

All experiments were approved by the Institutional Animal Care and Use Committee (IACUC) at the Rutgers New Jersey Medical School, under protocol number 13086D1016, following guidelines of the Animal Welfare Act, The Institute of Laboratory Animal Resources Guide for the Care and Use of Laboratory Animals, and Public Health Service Policy. Swiss-webster mice (6-8 weeks, female, Taconic Biosciences) were utilized for all experiments.

Conditional mutagenesis and parasite transfection

The CDPK4 cKO targeting vector was constructed by cloning (In-Fusion, Clontech) three PCR products into a vector that carries two FRT sites and the human dihydrofolate reductase expression cassette (Falae *et al.*, 2010). Primers CTTGCATGCGCGGCCGCGTCTTTTACCATTTCTACAAT and TCGCCCTTATGCGG-CCGCCTTTAACTTTCCTATATTTTATGC were used to amplify an approximately 1.0 kB fragment upstream of the 5'UTR of PbCDPK4 (PBANKA_061520). The product was cloned into the vector linearized with NotI. Primers TAGGAACTTCCTCGAGTACA-TATGTTCATATTAAGAAA and CTGGGCTGCACTCGAGAATAAATGAGTATTTAAAA-TATATAGG were used to amplify a 2.6 kB fragment encompassing the 5'UTR, exons 1-2 and 3'UTR of PbCDPK4. It was inserted into the previously generated plasmid using XhoI. Primers CTAGAGGATCCCCGGGTACCAATTATATATATGTATATAGTGTACG-TTG and CCATGATTACGAATTCTTGTATCATGTATATTCATGTTA were used to amplify a 0.5 kB fragment that was inserted into the previously derived plasmid digested with KpnI and EcoRI. The final insert was released from the targeting construct using NdeI and EcoRI. Transfections of TRAP/FlpL parasites (Panchal *et al.*, 2012) were carried out using standard methodology. Transfected parasites were selected using pyrimethamine and cloned by limiting dilution. Integration into the genome was assayed using primers P1 (CTTGCATGCGCGGCCGCGTCTTTTACCATTTCTACAAT) and P2 (CCATGATTACGAATTCTTGTATCATGTATATTCATGTTA), which amplify a 5.64 kB product in the presence of integration and a 4.14 kB product in the absence of integration. Integration was verified using Southern blotting of NdeI-digested genomic

DNA followed by hybridization with a dioxygenin-labeled probe (DIG High Prime DNA labeling and detection kit, Roche Applied Sciences) following the manufacturer's protocol.

Transgenic PKG-HA and PKG $T_{619}Q$ parasites were constructed in ANKA strain 507cl1 which expresses GFP under the control of the strong constitutive eef1α promoter that is active throughout the *P. berghei* life-cycle. Transfections and genotypic analysis were carried out essentially as described previously [8].

Mosquito Infections

Anopheles stephensi mosquitoes were fed on infected Swiss-Webster mice. Mosquitoes infected with PKG-HA and PKG T619Q-HA parasites were maintained at 20 °C. Sporozoites were obtained at days 18-21 post-feeding through dissections of salivary glands. Mosquitoes infected with CDPK4 cKO and TRAP/FlpL parasites were maintained at 20° C until day 11 post bloodmeal and transferred to 25 °C thereafter. Sporozoites were obtained at days 21–26 post-bloodmeal.

Sporozoite invasion, liver stage infection and merosome formation

HepG2 cells (obtained from ATCC) were cultured in Dulbecco's Modified Eagle Medium (high glucose) supplemented with 10% FCS. Cells were seeded on collagen-coated multi-chambered slides for overnight growth at 37 °C prior to addition of sporozoites. Invasion assays were performed using cells at 90% confluency and infection assays were perfomed with cells at 50% confluency, essentially as previously described (Sinnis *et al.*, 2013). For invasion assays, cells were fixed in 4% paraformaldehyde 2 h after addition of sporozoites (1-2x10^4). Cells were blocked in 1% BSA/PBS before incubation with anti-CS antibody (3D11, 1 µg/mL) and anti-mouse Alexa488. Cells were then permeabilized with cold methanol, blocked, incubated with 3D11 and anti-mouse Alexa594. Extracellular sporozoites were quantified by determining the number of sporozoites that stained exclusively with anti-mouse Alexa594. The total sporozoite number was quantified by determining the number of sporozoites stained with both Alexa488 and Alexa594. For infection assays, cells infected with sporozoites (1-2x10^4) were fixed in 4% paraformaldehyde 24-48 h p.i.. Liver stages were detected in immunofluorescence assays using a monoclonal antibody against PbHSP70 (10 µg/mL). For merosome assays, cells were seeded on collagen-coated coverslips for overnight growth before infection with 5-8x10^4 sporozoites. Media was changed every 12 h. The number of merosomes released in the media was quantified at 66-72 h p.i. using a hemocytometer. To determine the pre-patent period of infection was Swiss-webster mice (female, 6-8 weeks, Taconic Biosciences) were injected intravenously with sporozoites. Parasitemia was determined daily either through microscopic counting of Giemsa-stained blood smears or FACS analysis.

Immunofluorescence for protein detection

Purified sporozoites (Kennedy *et al.*, 2012) were air-dried on poly-lysine coated slides, fixed in 4% paraformaldehyde for 15 min and permeabilised in 0.5% TritonX-100 for 5 min. They were blocked in 3% BSA/PBS for 1 h prior to incubation with antibodies. Liver stages were fixed with 4% paraformaldehyde for 15 min, permeabilized with cold

methanol for 15 min, blocked in 3% BSA/PBS for 1 h prior to incubation with antibodies. Primary antibodies were anti-HA (mouse monoclonal, Covance, 10 µg/mL), anti-CS (mouse monoclonal 3D11, 1 µg/mL), anti-HSP70 (1 µg/mL, (Tsuji *et al.*, 1994)), anti-PKG (polyclonal antisera raised against a peptide containing amino acids 989 to 1003 of PbPKG), anti-merosome antibody (rat, 1:100, a kind gift of Dr. Volker Heussler), anti-TgCDPK1 (polyclonal antisera, a kind gift of Dr. Conrad Beckers), which recognizes PbCDPK4 (Billker *et al.*, 2004). Secondary antibodies were anti-mouse Alexa488, anti-mouse Alexa594, anti-rabbit Alexa594 and anti-rabbit Alexa488. All secondary antibodies were purchased from Santa Cruz Biotechnology and used at 0.7 µg/mL). Images were collected on a Nikon A1R laser scanning confocal microscope using 60X/NA1.4 oil objective. For quantification of signal intensities, all images in a given experiment were captured using the same excitation laser intensity and detector gain settings. A region-of-interest comprising a single sporozoite was automatically selected and the mean background corrected fluorescence intensity of Alexa488 and Alex594 within that region-of-interest was measured using the Nikon NIS Elements Advanced Research software. The average ratio of Alexa488 and Alexa594 signal intensities was determined for a given sporozoite population.

Western Blot for protein detection

Protein lysates of schizont stage parasites were examined by SDS-PAGE using anti-HA (mouse monoclonal, Covance, 2 µg/mL) and anti-HSP-70 (0.4 µg/mL (Tsuji *et al.*, 1994)) antibodies followed by detection using chemiluminescence (SuperSignal™ West Femto substrate, ThermoFisher Scientific) following the manufacturer's protocol. Signal intensities were quantified using Image J software.

Compound treatment

The effect of compounds on sporozoite infectivity was determined by adding sporozoites to HepG2 cells in the presence of appropriate concentrations of TSP, 1294, both together or vehicle alone. Compound-containing media was replaced with compound-free medium at 14 h p.i. The effect of compounds on sporozoite invasion was determined by treating sporozoites for 30 min on ice in a volume of 20 µL prior to addition to HepG2 cells in a volume of 200 µL. Fresh compound-free media was added 2 h later. Liver stages present at 44-48 h p.i. were detected as described above.

Imaging sporozoite motility

Sporozoites were filmed on a Nikon A1R laserscanning confocal microscope using a 20X/NA0.75 objective at 37°C in a 96-well plate with an optical bottom (. Dissected sporozoites, in RPMI and 3% BSA, were incubated with appropriate compounds for 15 min on ice prior to centrifugation for 3 minutes at 4°C. Movies were recorded over 90 frames at 1Hz. Image acquisition and analysis was performed using NIS Elements software from Nikon. Fluorescence intensity projections were processed using NIS Elements and movement patterns were determined through visual inspection of individual sporozoites.

Statistical analysis

Invasion and infection of HepG2 cells by sporozoites was examined using the Kruskal-Wallis test (Kruskal, 1952). The Kruskal-Wallis test is a non-parametric test appropriate for comparing a continuous outcome measured in two or more groups. It is the non-parametric analog of a ANOVA test when there are 3 or more groups; and analog of t-test when there are only 2 groups.

Figure Legends

Figure 1: **PbPKG is expressed in pre-erythrocytic stages.** A) HA-tagged PbPKG (PKG-HA) was localized in sporozoites and liver stages using immunostaining with an anti-HA antibody. All sporozoites and liver stages express HA-tagged PKG in the cytoplasm. The anti-Exp1 antibody recognizes Exp1, a resident protein of the parasitophorous vacuole membrane in liver stage parasites. Merged panels include DAPI for nuclear localization. B) PbPKG cKO sporozoites contain PbPKG protein. Polyclonal antisera against PbPKG (amino acids 988-1001) were used to localize PbPKG in sporozoites and liver stages from wildtype and PbPKG cKO parasites. Sporozoites were co-stained with an antibody against the circumsporozoite protein (CS). Liver stages were co-stained with an antibody against Heat Shock Protein 70 (HSP70). Merged images include DAPI as a nuclear marker. PKG protein is readily detected in PbPKG cKO sporozoites but is decreased significantly in PbPKG cKO liver stages.

Figure 2: PbPKG is required for sporozoite motility, invasion and merosome formation. A) Inhibition of PKG activity blocks sporozoite infectivity. HepG2 cells were infected with PKG-HA or PKG T_{619}Q-HA sporozoites in the presence of TSP and compound exposure was maintained for 0-14 h p.i. (solid lines). Liver stages were quantified at 44 h p.i.. Results shown are from a representative experiment (mean of 4 replicates $\pm$ standard deviation). The experiment was repeated thrice. B) PKG is required for sporozoite motility. Motility of PKG-HA or PKG T_{619}Q-HA sporozoites was examined in the presence of TSP using live imaging. Movement patterns of individual sporozoites were assigned manually. The percentage ($\pm$ standard error, n = 2 experiments) of gliding sporozoites was determined. Data were analyzed using chi-square tests, ***$p<0.005$. For both PKG-HA and PKG T_{619}Q-HA sporozoites, 100% is the proportion of sporozoites that glide in the absence of TSP. C) Inhibition of PKG activity prevents merosome formation. HepG2 cells infected with PKG-HA or T_{619}Q-HA sporozoites were treated with TSP at 24-65 h p.i.. Merosomes released into the media at 66 h p.i. were quantified. Merosomes in TSP-treated cultures were quantified as a percentage of merosomes released in untreated cultures (mean of 3 replicates $\pm$ standard deviation).

Figure 3: Conditional mutagenesis of PbCDPK4 using the FlpL-FRT system. A) PbCDPK4 is expressed in the cytoplasm of pre-erythrocytic stages. Immunofluorescence assays using anti-TgCDPK1 antibodies were used to determine PbCDPK4's subcellular localization in WT sporozoites and liver stages. The anti-CS antibody recognizes CS, a sporozoite membrane protein. Anti-HSP70 recognizes HSP70, a cytoplasmic protein. PbCDPK4 co-localizes with HSP70, a cytoplasmic

marker in liver stage parasites. Merged panels include DAPI for nuclear localization. B) Modification of CDPK4 open reading frame through addition of two FRT sites (green arrows) and a hDHFR expression cassette (red box) in FlpL-expressing parasites. The CDPK4 ORF is excised during sporogony in the mosquito midgut generating CDPK4 cKO sporozoites in the mosquito salivary glands. C) PCR analysis demonstrates excision of CDPK4 in blood stage parasites obtained from cKO sporozoite infection. Amplification products of primers P1 and P2 from genomic DNA of (1) WT blood stages, (2) parasites obtained after integration of the targeting plasmid, (3) blood stages resulting from infection with CDPK4 cKO sporozoites. D) Southern blot analysis confirms loss of CDPK4 in blood stage parasites obtained from cKO sporozoites. NdeI-digested genomic DNA obtained from (1) blood stages resulting from infection by CDPK4 cKO sporozoites (2) blood stages obtained after integration of the targeting construct (3) WT parasites. The probe demonstrates non-specific hybridization to a 2.3kb fragment (indicated by an asterisk) in genomic DNA from all parasite populations.

Figure 4: CDPK4 plays a role in sporozoite invasion. A) Invasion by CDPK4 cKO sporozoites of HepG2 cells and intracellular development of CDPK4 cKO liver stages (LS) relative to FlpL-expressing sporozoites. Equal numbers of control (FlpL-expressing parent line) and CDPK4 cKO sporozoites were used to assay invasion and intracellular development. Sporozoite invasion was quantified by determining the fraction of sporozoites that are intracellular 2 h after addition to HepG2 cells. Intracelluar development of sporozoites was quantified by determining the number LS at 24 h p.i and 48 h p.i. Results shown are from a representative experiment (average of 4 replicates $\pm$ standard deviation). The experiment was performed four times. Parasite egress was examined by quantifying merosomes released at 65-72 h p.i. relative to the number of LS formed at 48 h p.i.. To compensate for their decreased invasion, twice as many CDPK4 cKO sporozoites as FlpL-expressing sporozoites were used to infect HepG2 cells. Results shown are from a representative experiment (mean of 3-4 replicates $\pm$ standard deviation). The experiment was performed thrice. Data were analyzed using Kruskal-Wallis test, *$p<0.05$. B) Inhibition of CDPK4 activity decreases sporozoite motility. PbGFP-Luc sporozoites were filmed in the presence of 1294. The percentage of sporozoites that glide was determined. The percentage ($\pm$ standard error, n = 2 experiments) of gliding sporozoites was determined. Data were analyzed using chi-square tests, ***$p<0.005$. 100% is the proportion of sporozoites that glide in the absence of 1294.

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Supporting Information

Supplementary Figure 1. Generation and characterization of PKG-HA and PKG T_{619}Q-HA lines in PbANKA 507cl1. Transgenic PKG-HA and PKG T_{619}Q-HA lines were generated in PbANKA 507cl1 as previously described [8]. A) Targeting vector used to modify the *pkg* locus and oligonucleotides used for PCR are shown. B) PCR products used to genotype genomic DNA obtained from transfected parasites are shown. C) Sequence chromatograms confirm the T_{619}Q mutation. D) Expression of PKG in PKG-HA (lanes 1, 3) and PKG T_{619}Q-HA (lanes 2, 4) in erythrocytic stage parasites was examined using Western blot analysis with an anti-HA (α-HA) antibody. *P. berghei* HSP70 (α-HSP70) was used as loading control. The relative signal ratio of α-HA and α-HSP70 in each lane is given below. Lanes 1-2 contain $4x10^7$ parasite equivalents and lanes 3-4 contain $1x10^8$ parasite equivalents. E) Erythrocytic stage infection of PKG-HA and PKG T_{619}Q-HA parasites was examined by determining the parasitemias of mice infected intravenously with 1000 erythocytic stage parasites from each line. Percentage of infected cells was determined daily through FACS analysis. F) Decreased invasion by PKG T_{619}Q-HA sporozoites results in reduced infectivity. Sporozoite invasion was quantified by determining the fraction of sporozoites that are intracellular 2 h post-addition to HepG2 cells. Results shown are from a representative experiment (mean of

4 replicates ± standard deviation). The experiment was repeated twice. Data were analyzed using Kruskal-Wallis test, *p<0.05. Invasion by PKG T_{619}Q-HA sporozoites is shown as a percentage of invasion by PKG-HA (control) sporozoites. Intracellular development of sporozoites was quantified by determining the number of liver stage (LS) parasites at 24 h p.i and 48 h p.i.. Results shown are from a representative experiment (mean of 4 replicates ± standard deviation). Each experiment was repeated 2-4 times. Data were analyzed using Kruskal-Wallis test, *p<0.05. The number of LS formed by PKG T_{619}Q-HA sporozoites is shown as a percentage of LS formed by PKG-HA sporozoites. G) Expression of PKG in PKG-HA and PKG T_{619}Q-HA sporozoites was examined in immunofluorescence assays with an anti-HA (α-HA) antibody. As control, GFP expression was examined using an anti-GFP antibody (α-GFP). The ratio of mean anti-HA and anti-GFP fluorescence was determined for each sporozoite. Results shown are from a representative experiment. Data were analyzed using a log transformed unpaired t-test, ***p<0.0005.

Supplementary Figure 2. Maximum intensity projections of sporozoite motility patterns. Examples of motility patterns of PKG-HA and PKG T_{619}Q-HA sporozoites in the absence or presence of TSP. Large arrowheads: sporozoites that displayed gliding motility during the entire imaging period; small arrowheads: sporozoites that displayed 'waving'; small arrows: 'complex' motility; large arrows: drifting sporozoites.

Supplementary Table 1. Effect of PKG inhibition on sporozoite infectivity. The table displays the effects of TSP treatment on sporozoite motility, invasion and liver stage development.

Supplementary Table 2. Effect of CDPK4 inhibition on sporozoite infectivity. The table displays the effects of genetic and chemical inhibition of CDPK4 on sporozoite motility, invasion, intracellular liver stage development and hepatocyte egress.

Figure 1

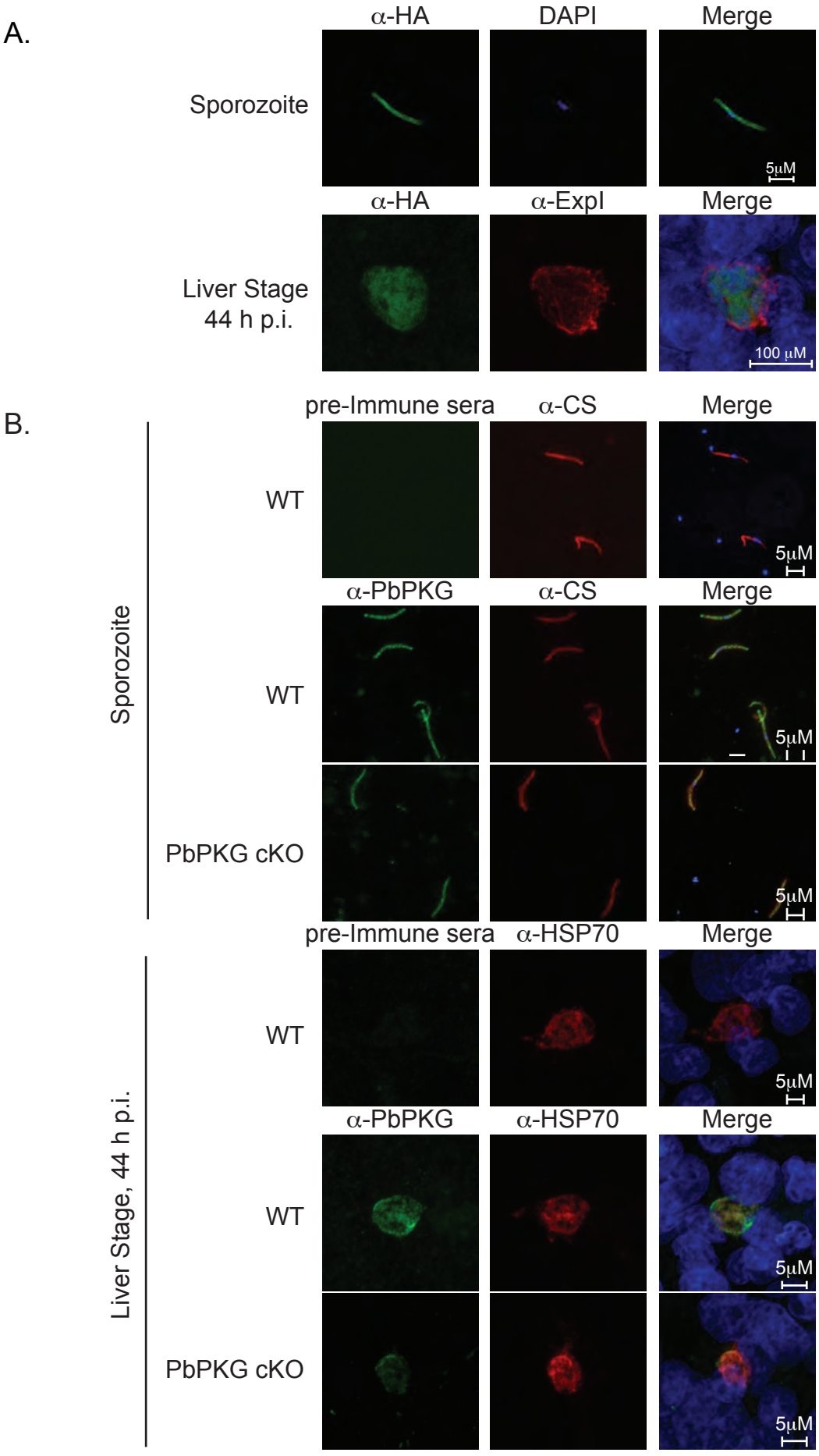

A.
α-HA
DAPI
Merge
Sporozoite
5μM
α-HA
α-Expl
Merge
Liver Stage
44 h p.i.
100 μM
B.
pre-Immune sera
α-CS
Merge
WT
5μM
α-PbPKG
α-CS
Merge
WT
5μM
PbPKG cKO
5μM
Sporozoite
pre-Immune sera
α-HSP70
Merge
WT
5μM
α-PbPKG
α-HSP70
Merge
WT
5μM
PbPKG cKO
5μM
Liver Stage, 44 h p.i.

Figure 2

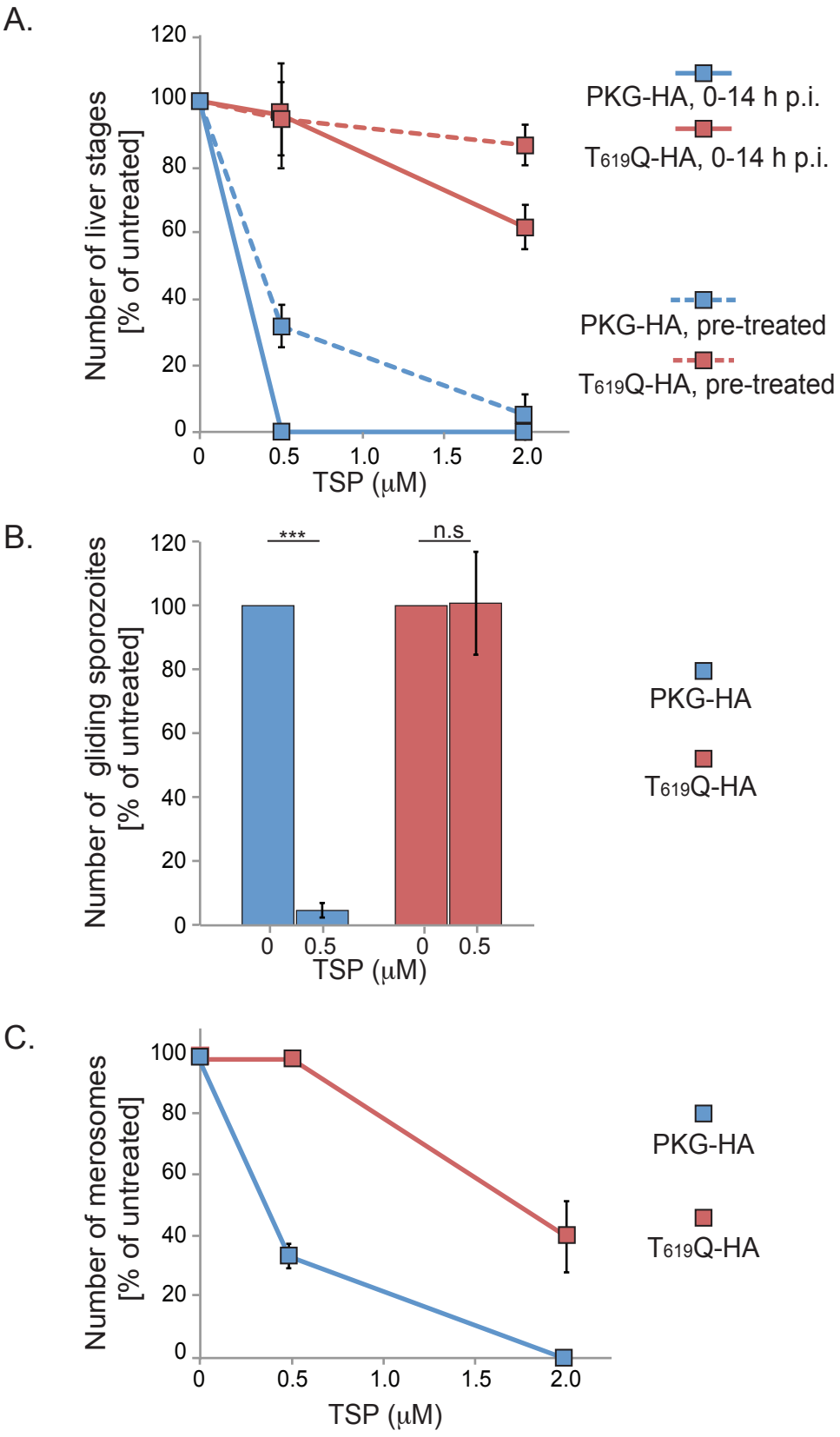

A.
Number of liver stages [% of untreated]
120
100
80
60
40
20
0
0
0.5
1.0
1.5
2.0
TSP (µM)
PKG-HA, 0-14 h p.i.
T619Q-HA, 0-14 h p.i.
PKG-HA, pre-treated
T619Q-HA, pre-treated
B.
Number of gliding sporozoites [% of untreated]
***
n.s
0
0.5
0
0.5
TSP (µM)
PKG-HA
T619Q-HA
C.
Number of merosomes [% of untreated]
TSP (µM)
PKG-HA
T619Q-HA

Figure 3

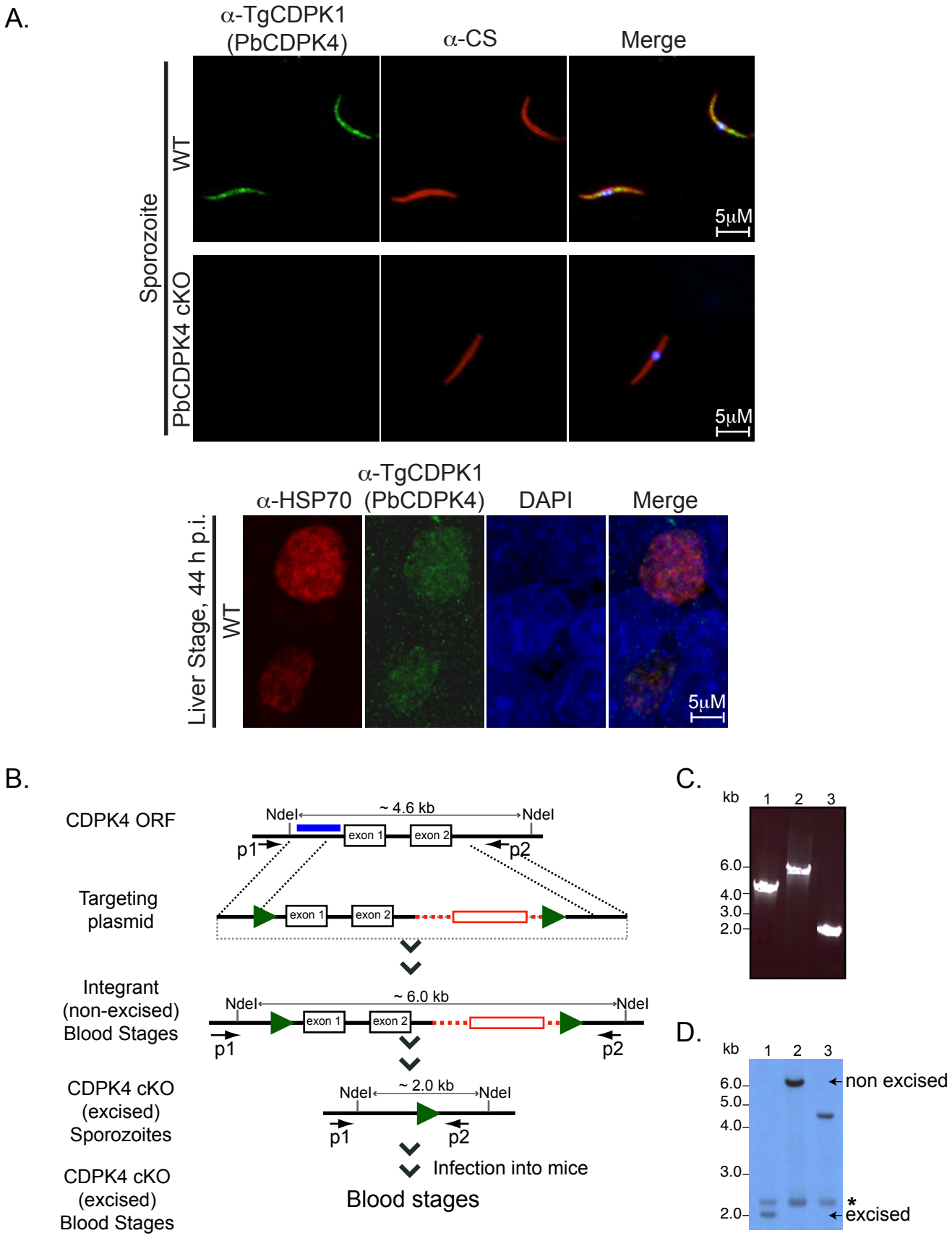
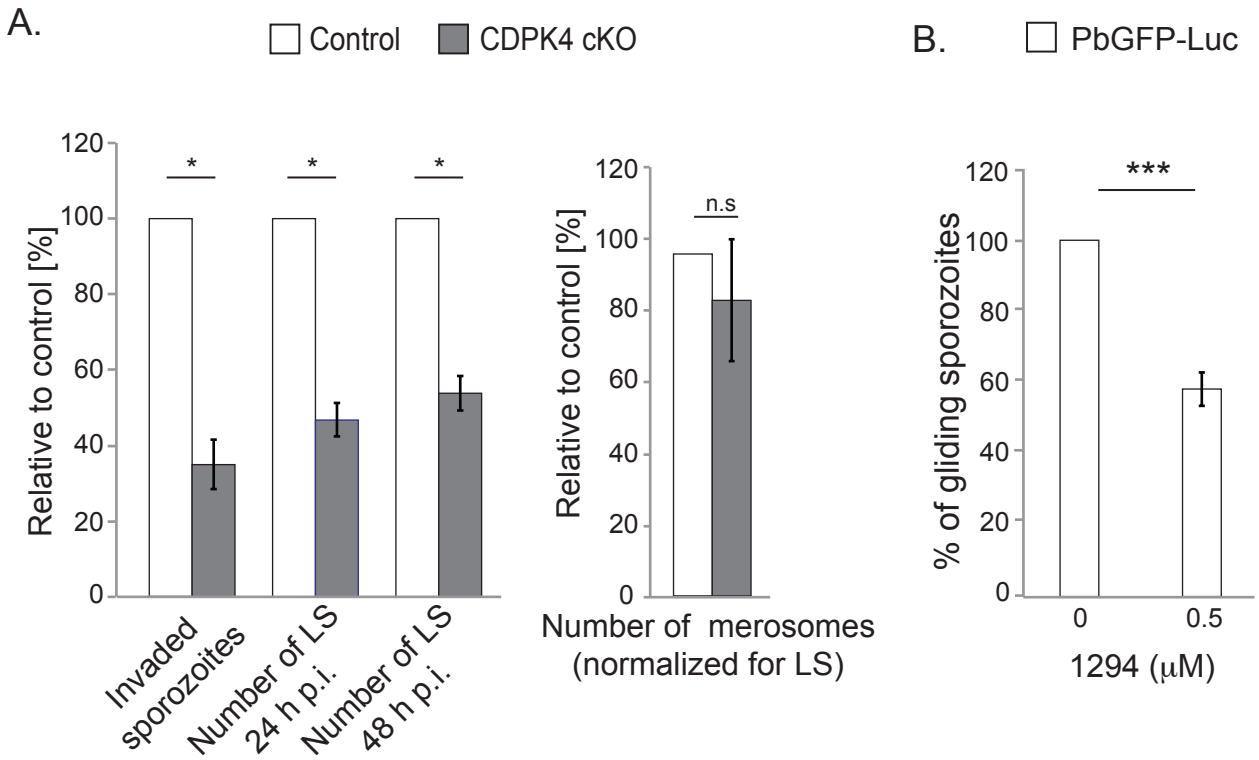
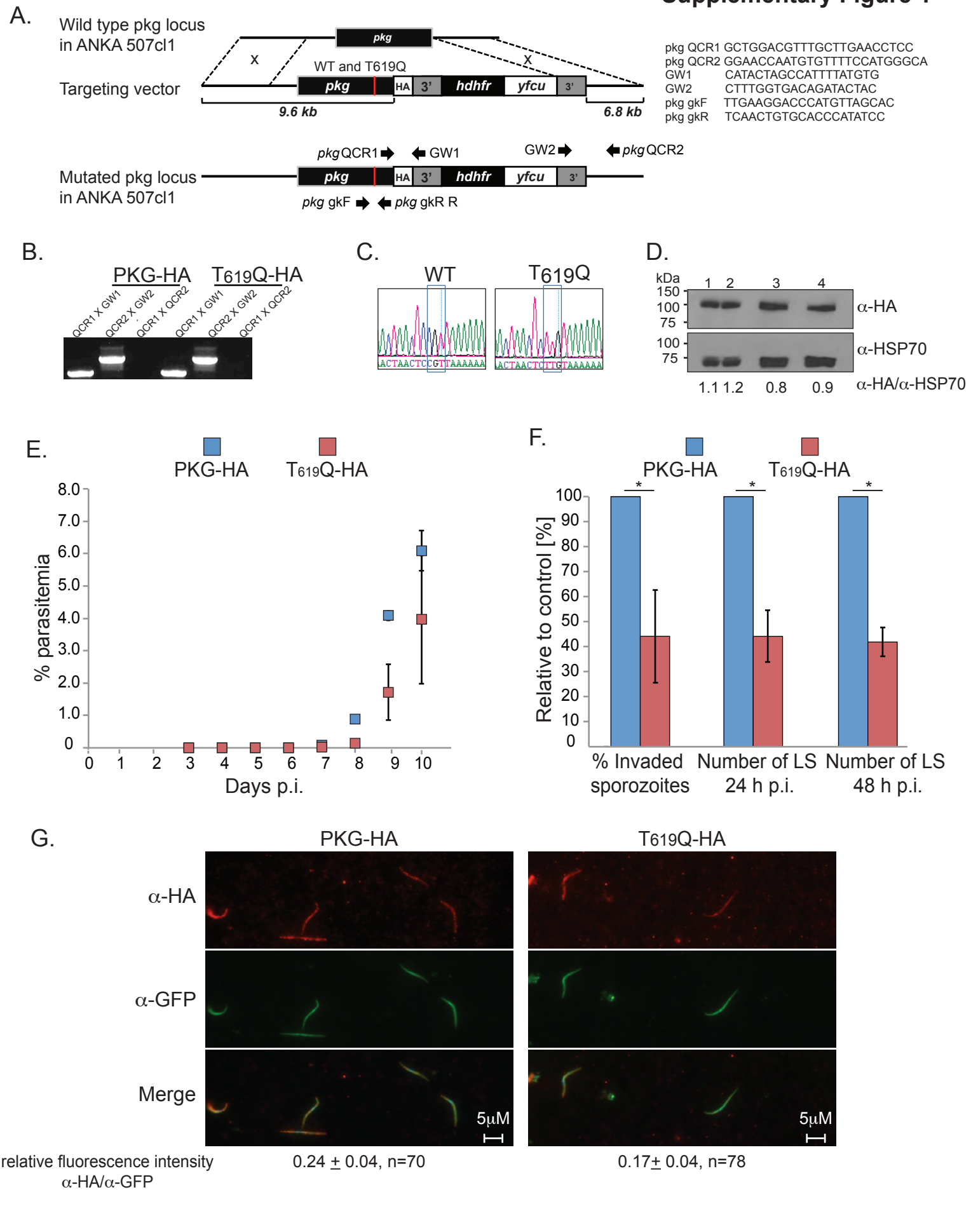

A.
α-TgCDPK1 (PbCDPK4)
α-CS
Merge
Sporozoite
WT
PbCDPK4 cKO
5μM
5μM
α-TgCDPK1 (PbCDPK4)
α-HSP70
DAPI
Merge
Liver Stage, 44 h p.i.
WT
5μM
B.
CDPK4 ORF
NdeI
~ 4.6 kb
NdeI
exon 1
exon 2
p1
p2
Targeting plasmid
exon 1
exon 2
Integrant (non-excised) Blood Stages
NdeI
~ 6.0 kb
NdeI
exon 1
exon 2
p1
p2
CDPK4 cKO (excised) Sporozoites
NdeI
~ 2.0 kb
NdeI
p1
p2
Infection into mice
CDPK4 cKO (excised) Blood Stages
Blood stages
C.
kb
1
2
3
6.0
4.0
3.0
2.0
D.
kb
1
2
3
6.0
5.0
4.0
3.0
2.0
non excised
*
excised

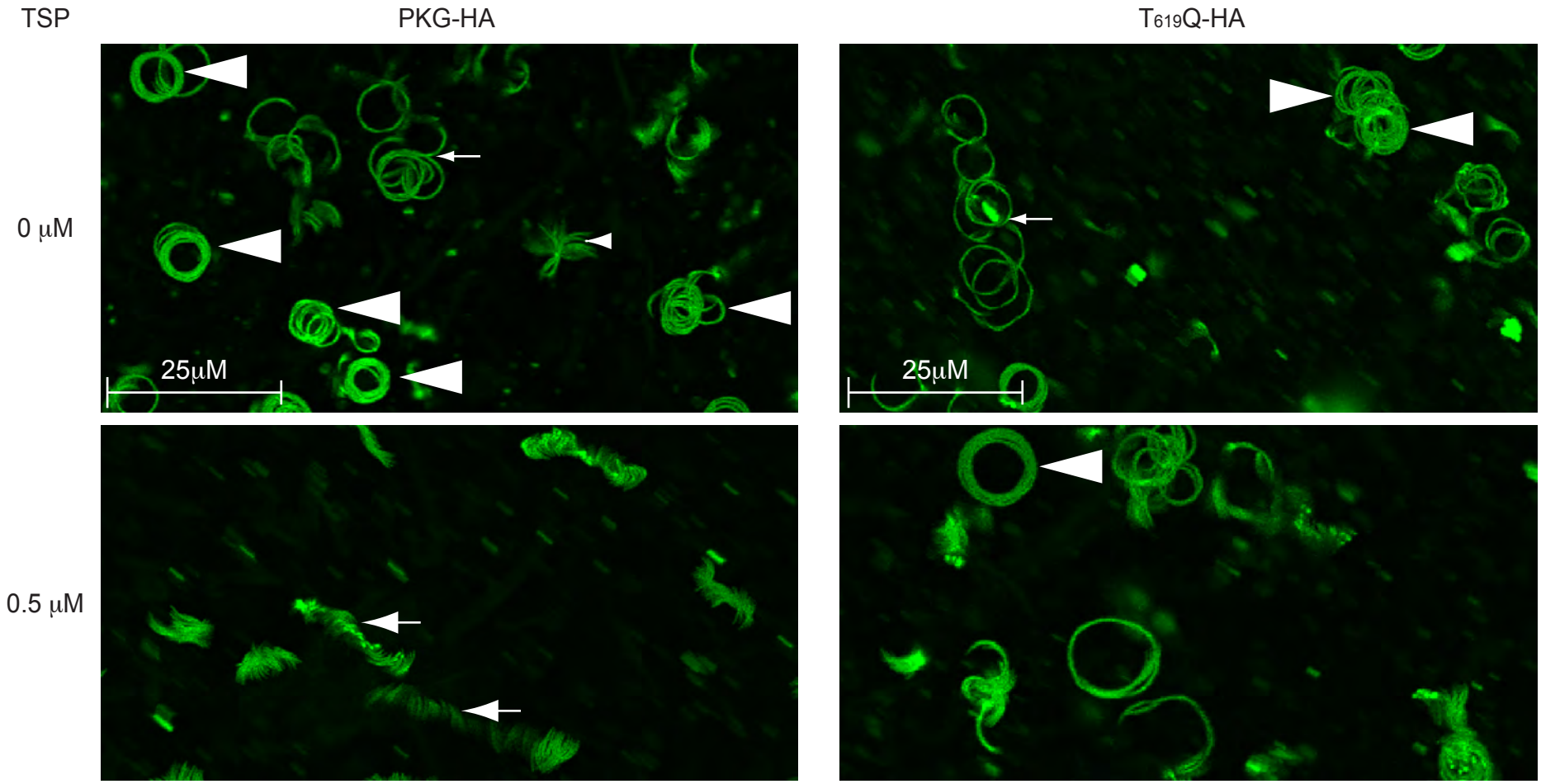
Figure 4


A.
Control
CDPK4 cKO
Relative to control [%]
120
100
80
60
40
20
0
*
*
*
Invaded sporozoites
Number of LS 24 h p.i.
Number of LS 48 h p.i.
n.s
Number of merosomes (normalized for LS)
B.
PbGFP-Luc
% of gliding sporozoites
***
0
0.5
1294 (μM)

Supplementary Figure 1



Supplementary Figure 2



Supplementary Table 1

A) Sensitivity to TSP: 0-14h treatment

[TSP]	PKG-HA	T619Q-HA
Experiment 1 (n=3)	# of LS, 40h p.i (mean ± SD)	
0 µM	224 ± 12	150 ± 6
0.5 µM	0 ± 0	143 ± 16
2 µM	0 ± 0	89 ± 7
10 µM	0 ± 0	4 ± 0.6
Experiment 2 (n=4)		
0 µM	328 ± 41	157 ± 38
0.5 µM	0 ± 0	199 ± 13
2 µM	0 ± 0	149 ±32
10 µM	0 ± 0	24 ± 6
Experiment 3 (n=4)		
0 µM	411 ± 10	217 ± 9
0.5 µM	0 ± 0	295 ± 18
2 µM	0 ± 0	290 ± 16
10 µM	0 ± 0	44 ± 9

Sensitivity to TSP: pre-treatment

[TSP]	PKG-HA	T619Q-HA
Experiment 1 (n=4)	# of LS, 40h p.i (mean ± SD)	
0 µM	355 ± 15	117 ± 3
0.5 µM	114 ± 6	110 ± 11
2 µM	18 ± 6	101 ± 6
Experiment 2 (n=4)		
0 µM	615 ± 47	24.5 ± 1
2 µM	24.5 ± 1	77 ± 0.5

Sensitivity to TSP: 24-65h treatment

[TSP]	PKG-HA	T619Q-HA
Experiment 1 (n=3)	# of merosomes (mean ± SD)	
0 µM	10000 ± 589	3750 ± 884
0.5 µM	3333 ± 0	3750 ± 884
Experiment 2 (n=3)		
0 µM	2121 ± 1060	2500 ± 353
2 µM	0 ± 0	1000 ± 0.0

B) Effect of TSP on motility

Experiment 1

PKG-HA [TSP]	Gliding # of sporozoites	Drifting # of sporozoites	Waving # of sporozoites	Adherent # of sporozoites	Complex # of sporozoites	Total observed
0 µM	54	12	29	205	15	315
0.5 µM	4	4	53	43	93	197
T619Q-HA						
0 µM	13	7	69	257	2	348
0.5 µM	15	3	116	157	29	320

Experiment 2

PKG-HA						
0 µM	55	3	6	125	64	253
0.5 µM	0	11	49	9	181	250
T619Q-HA						
0 µM	27	3	18	126	98	272
0.5 µM	22	19	55	117	35	248

Experiment 1

PbGFP-Luc [TSP]	Gliding # of sporozoites	Drifting # of sporozoites	Waving # of sporozoites	Adherent # of sporozoites	Complex # of sporozoites	Total observed
0 µM	134	35	10	3	67	249
0.5 µM	83	55	18	11	39	206
2 µM	2	94	4	5	1	106

Experiment 2

PbGFP-Luc						
0 µM	104	15	26	14	37	196
0.5 µM	65	41	40	5	34	185
2 µM	0	74	70	7	0	151

C) % invaded sporozoites (mean ± SD)

	PKG-HA	T619Q-HA
Experiment 1 (n=4)	71 ± 7	31 ± 6
Experiment 2 (n=4)	9 ± 0.6	4 ± 0.7

Number of LS, 24h p.i (mean ± SD)

	PKG-HA	T619Q-HA
Experiment 1 (n=4)	456 ± 14	201 ± 21
Experiment 2 (n=4)	26 ± 0.7	16 ± 3

Number of LS, 48h p.i (mean ± SD)

	PKG-HA	T619Q-HA
Experiment 1 (n=4)	792 ± 18	332 ± 19
Experiment 2 (n=4)	411 ± 10	217 ± 9
Experiment 3 (n=4)	157 ± 10	78 ± 2.5

Supplementary Table 2

A) % invaded sporozoites (mean ± SD)

	Control	CDPK4 cKO
Experiment 1 (n=4)	49 ± 7	17 ± 1
Experiment 2 (n=4)	53 ± 3	35 ± 2
Experiment 3 (n=4)	56 ± 9	25 ± 0.4
Experiment 4 (n=4)	41 ± 4	19 ± 1

Number of LS, 24h p.i (mean ± SD)

	Control	CDPK4 cKO
Experiment 1 (n=4)	84 ± 3	39 ± 2
Experiment 2 (n=4)	823 ± 17	459 ± 17
Experiment 3 (n=4)	746 ± 12	458 ± 20
Experiment 4 (n=4)	135 ± 3	54 ± 5

Number of LS, 48h p.i (mean ± SD)

	Control	CDPK4 cKO
Experiment 1 (n=4)	159 ± 4	86 ± 4
Experiment 2 (n=4)	696 ± 41	410 ± 10
Experiment 3 (n=4)	756 ± 23	418 ± 9
Experiment 4 (n=4)	143 ± 14	47 ± 3

Number of merosomes, 65-72 h p.i (mean ± SD)

	Control	CDPK4 cKO
Experiment 1 (n=3)	3278 ± 1171	2833 ± 507
Experiment 2 (n=3)	10227 ± 693	12000 ± 2327
Experiment 3 (n=4)	2500 ± 833	3000 ± 1000

B) *In vivo* infection

	Control	CDPK4 cKO
	% parasitemia (mean ± SD)	
Experiment 1	n = 5	n = 4
Day 4	3.20E-03 ± 2.21E-03	7.25E-04 ± 7.25E-04
Day 6	5.84E-01 ± 2.66E-01	2.85E-01 ± 7.24E-02
Experiment 2	n = 10	n = 10
Day 4	1.02E-02 ± 6.09E-03	3.00E-04 ± 3.37E-04
Day 6	1.10E-01 ± 5.33E-02	3.95E-02 ± 1.92E-02

C) Effect of Compound 1294 on sporozoite motility

[1294]	Gliding	Drifting	Waving	Adherent	Complex	
Experiment 1	# of sporozoites	# of sporozoites	# sporozoi	# of sporozoites	# of sporozoites	Total observed
0 µM	136	9	14	74	92	325
2 µM	27	3	13	203	117	363
Experiment 2						
0 µM	134	119	13	169	9	444
0.5µM	106	193	23	216	32	570
Experiment 3						
0 µM	36	4	13	68	8	129
0.5µM	18	1	27	68	33	147

Figure 1

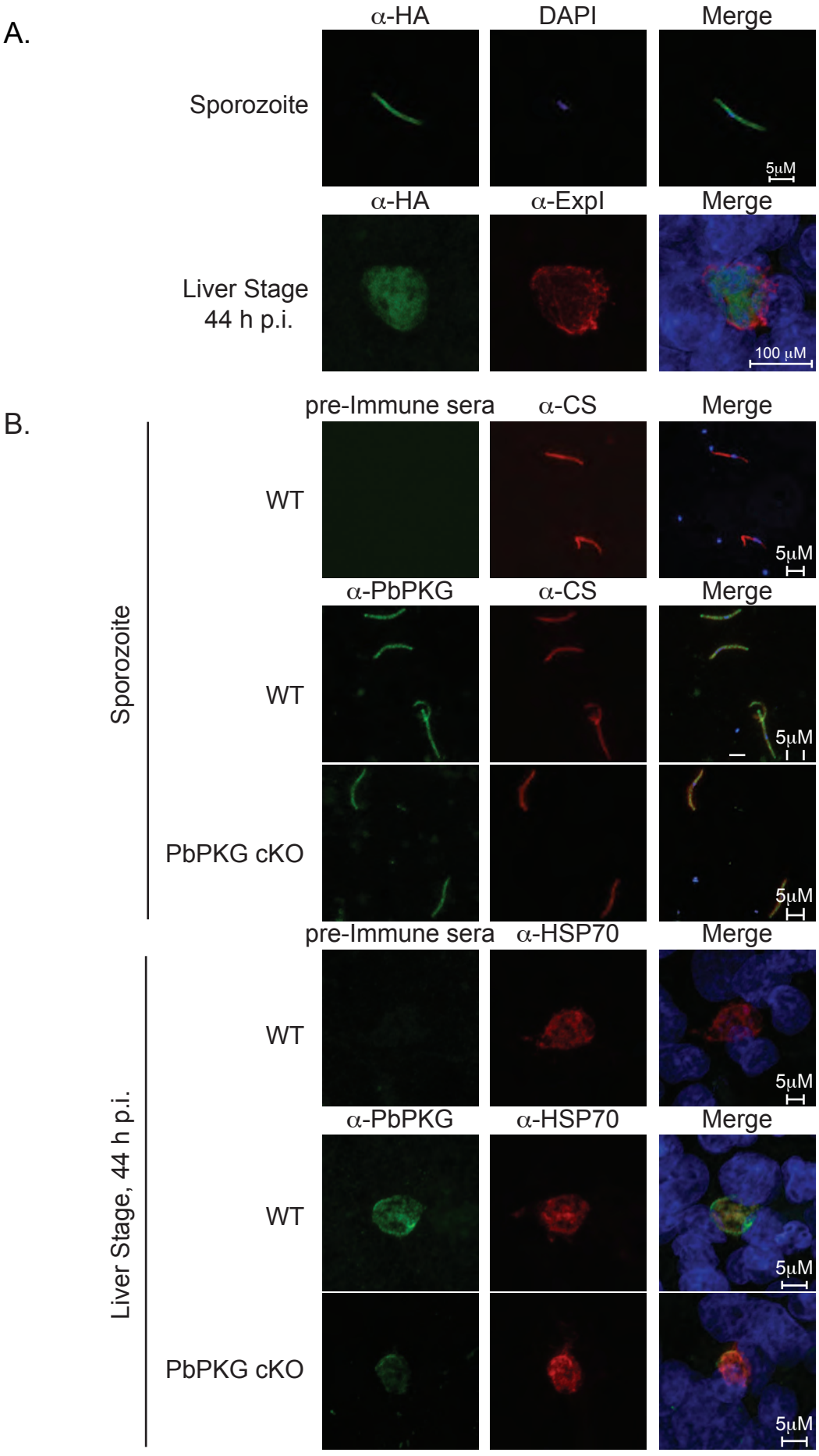

A.
α-HA
DAPI
Merge
Sporozoite
5μM
α-HA
α-Expl
Merge
Liver Stage
44 h p.i.
100 μM
B.
pre-Immune sera
α-CS
Merge
WT
5μM
α-PbPKG
α-CS
Merge
WT
5μM
PbPKG cKO
5μM
Sporozoite
pre-Immune sera
α-HSP70
Merge
WT
5μM
α-PbPKG
α-HSP70
Merge
WT
5μM
PbPKG cKO
5μM
Liver Stage, 44 h p.i.

Figure 2

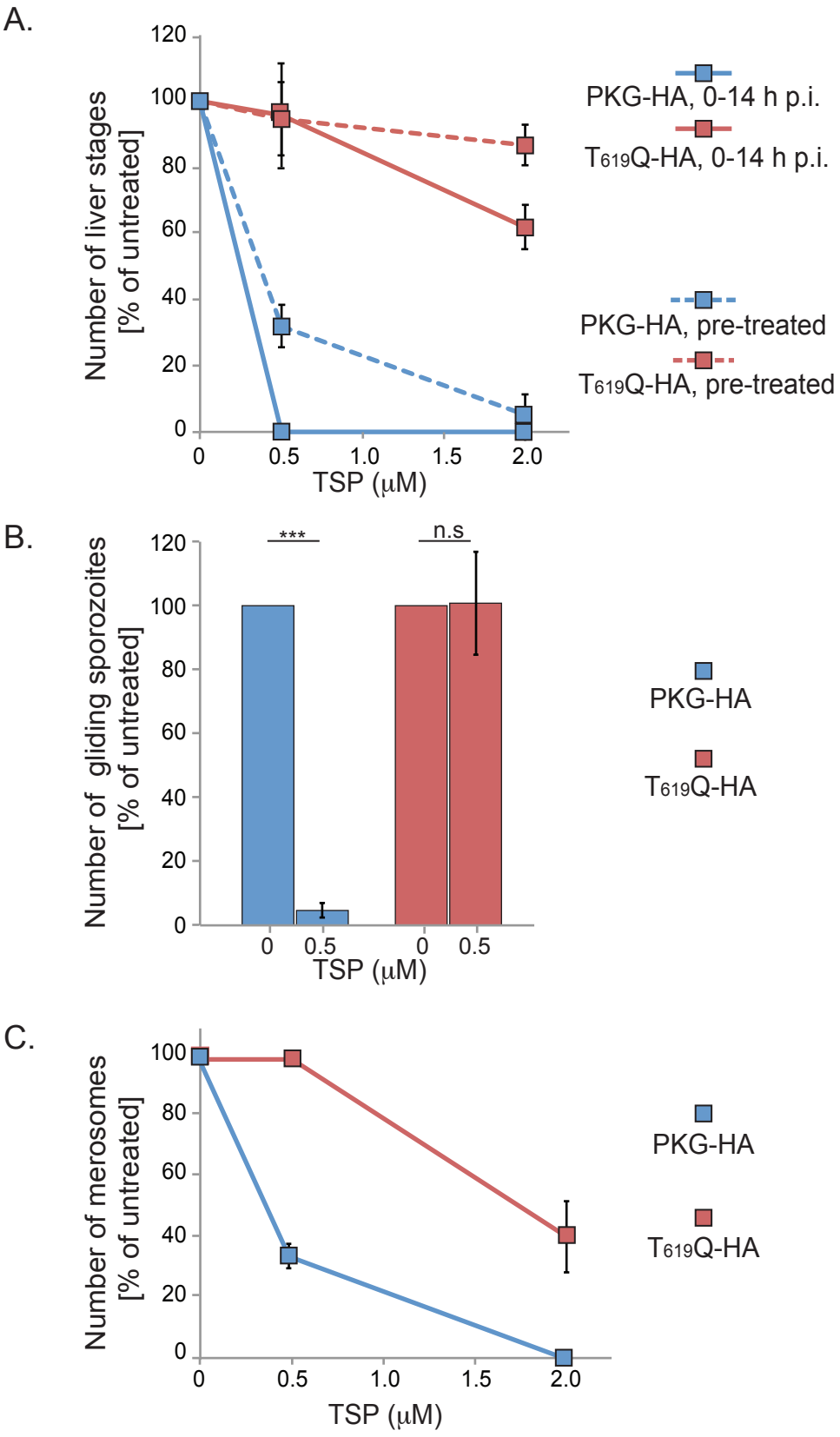

A.
Number of liver stages [% of untreated]
120
100
80
60
40
20
0
0
0.5
1.0
1.5
2.0
TSP (µM)
PKG-HA, 0-14 h p.i.
T619Q-HA, 0-14 h p.i.
PKG-HA, pre-treated
T619Q-HA, pre-treated
B.
Number of gliding sporozoites [% of untreated]
***
n.s
0
0.5
0
0.5
TSP (µM)
PKG-HA
T619Q-HA
C.
Number of merosomes [% of untreated]
TSP (µM)
PKG-HA
T619Q-HA

Figure 3

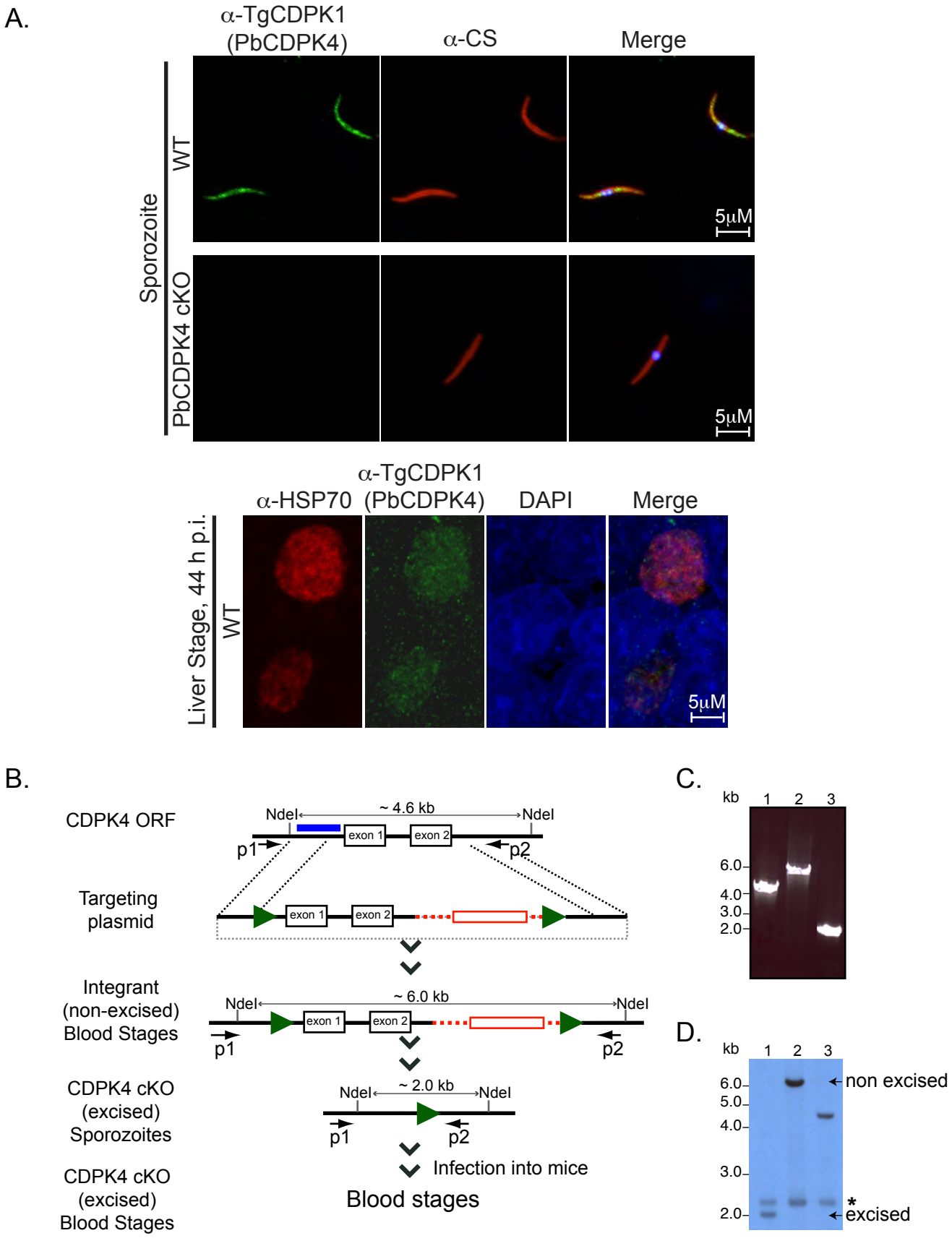
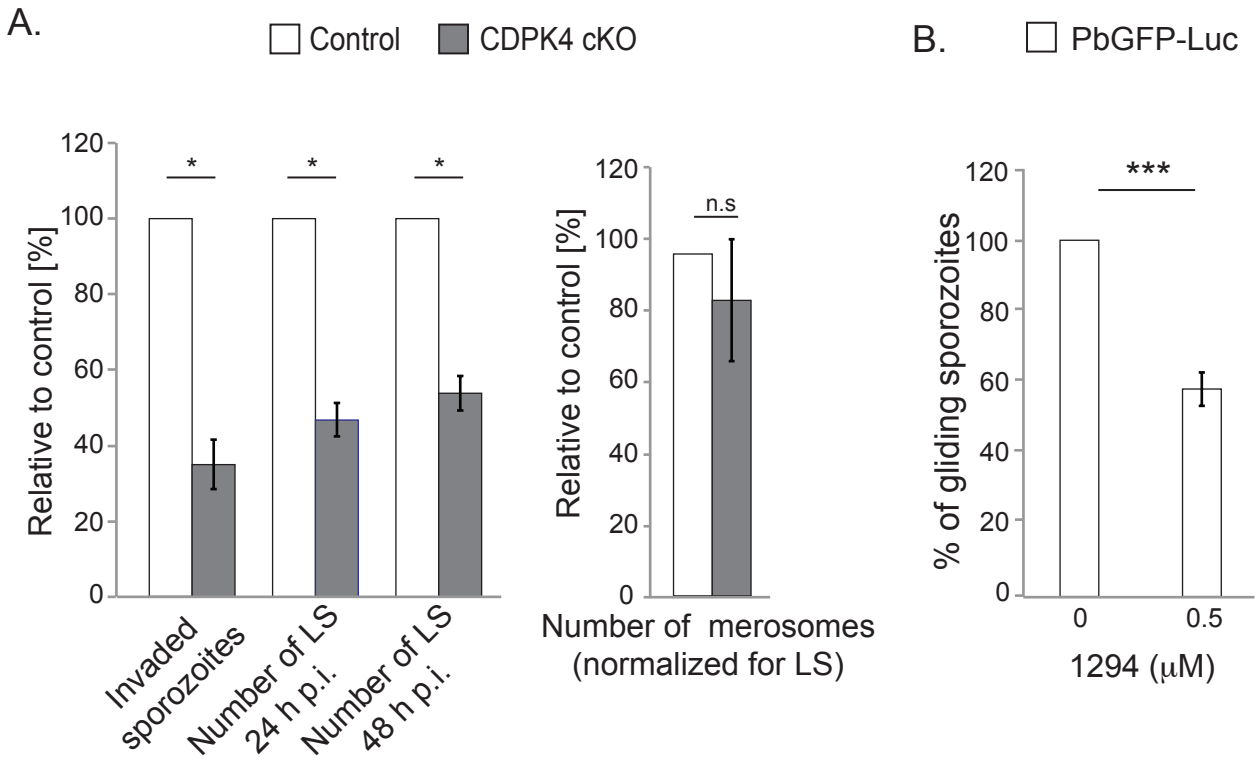
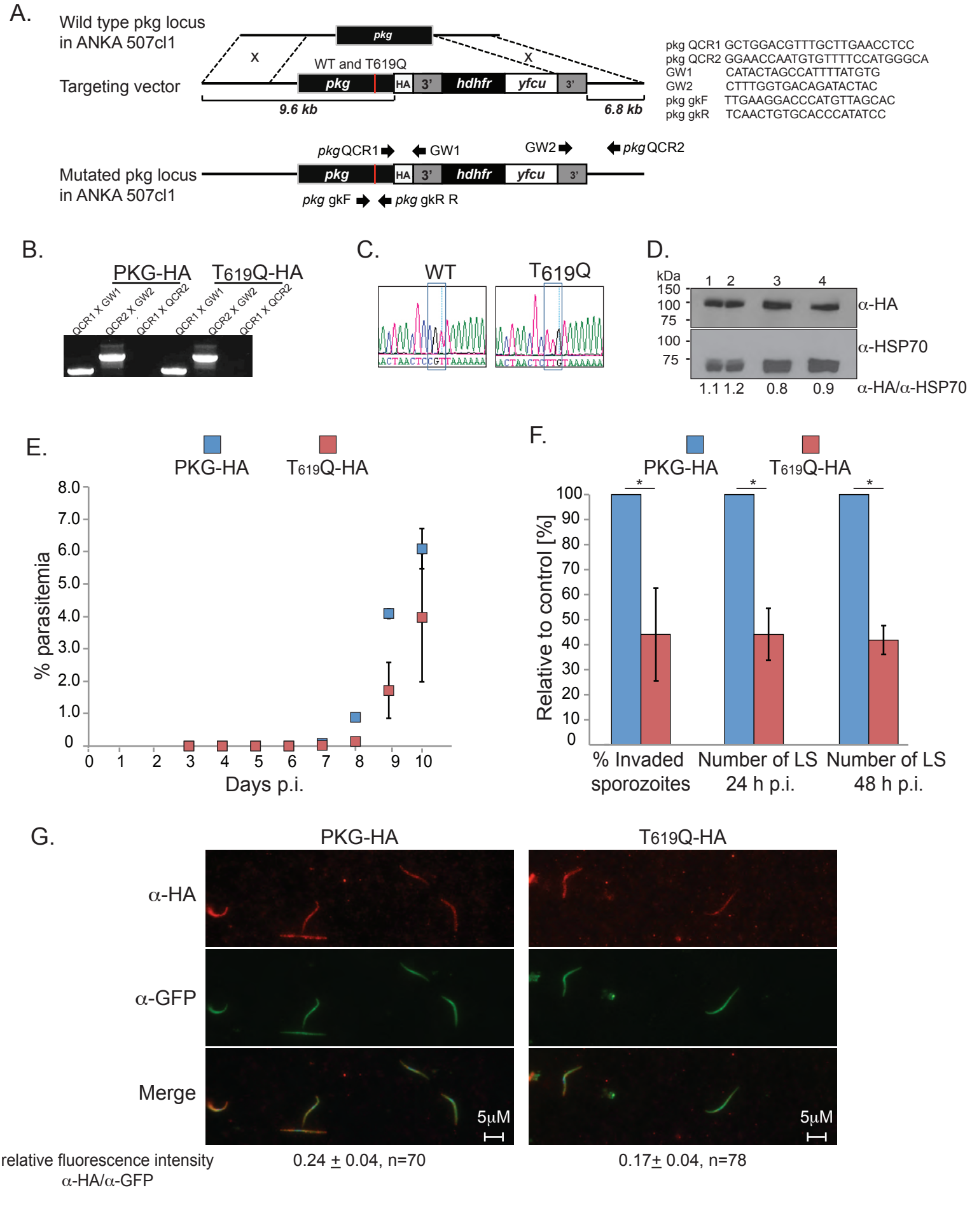

A.
α-TgCDPK1 (PbCDPK4)
α-CS
Merge
Sporozoite
WT
PbCDPK4 cKO
5μM
5μM
α-TgCDPK1 (PbCDPK4)
α-HSP70
DAPI
Merge
Liver Stage, 44 h p.i.
WT
5μM
B.
CDPK4 ORF
NdeI
~ 4.6 kb
NdeI
exon 1
exon 2
p1
p2
Targeting plasmid
exon 1
exon 2
Integrant (non-excised) Blood Stages
NdeI
~ 6.0 kb
NdeI
exon 1
exon 2
p1
p2
CDPK4 cKO (excised) Sporozoites
NdeI
~ 2.0 kb
NdeI
p1
p2
Infection into mice
CDPK4 cKO (excised) Blood Stages
Blood stages
C.
kb
1
2
3
6.0
4.0
3.0
2.0
D.
kb
1
2
3
6.0
5.0
4.0
3.0
2.0
non excised
*
excised

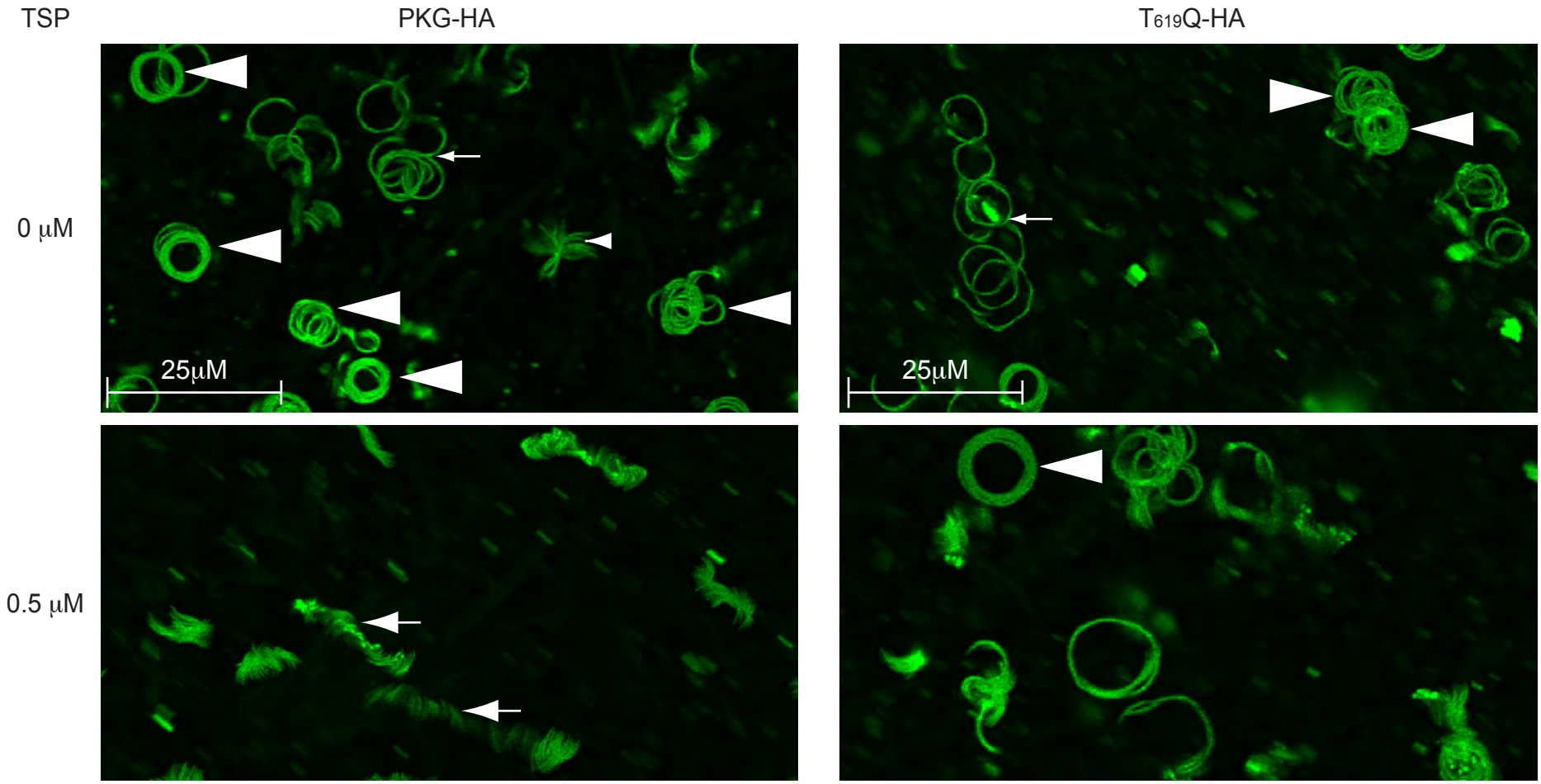
Figure 4


A.
Control
CDPK4 cKO
Relative to control [%]
120
100
80
60
40
20
0
*
*
*
Invaded sporozoites
Number of LS 24 h p.i.
Number of LS 48 h p.i.
n.s
Number of merosomes (normalized for LS)
B.
PbGFP-Luc
% of gliding sporozoites
***
0
0.5
1294 (μM)

Supplementary Figure 1



Supplementary Figure 2



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A) Sensitivity to TSP: 0-14h treatment

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Experiment 1 (n=3)	# of LS, 40h p.i (mean ± SD)	
0 µM	224 ± 12	150 ± 6
0.5 µM	0 ± 0	143 ± 16
2 µM	0 ± 0	89 ± 7
10 µM	0 ± 0	4 ± 0.6
Experiment 2 (n=4)		
0 µM	328 ± 41	157 ± 38
0.5 µM	0 ± 0	199 ± 13
2 µM	0 ± 0	149 ±32
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Experiment 3 (n=4)		
0 µM	411 ± 10	217 ± 9
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Sensitivity to TSP: pre-treatment

[TSP]	PKG-HA	T619Q-HA
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0 µM	355 ± 15	117 ± 3
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2 µM	18 ± 6	101 ± 6
Experiment 2 (n=4)		
0 µM	615 ± 47	24.5 ± 1
2 µM	24.5 ± 1	77 ± 0.5

Sensitivity to TSP: 24-65h treatment

[TSP]	PKG-HA	T619Q-HA
Experiment 1 (n=3)	# of merosomes (mean ± SD)	
0 µM	10000 ± 589	3750 ± 884
0.5 µM	3333 ± 0	3750 ± 884
Experiment 2 (n=3)		
0 µM	2121 ± 1060	2500 ± 353
2 µM	0 ± 0	1000 ± 0.0

B) Effect of TSP on motility

Experiment 1

PKG-HA [TSP]	Gliding # of sporozoites	Drifting # of sporozoites	Waving # of sporozoites	Adherent # of sporozoites	Complex # of sporozoites	Total observed
0 µM	54	12	29	205	15	315
0.5 µM	4	4	53	43	93	197
T619Q-HA						
0 µM	13	7	69	257	2	348
0.5 µM	15	3	116	157	29	320

Experiment 2

PKG-HA						
0 µM	55	3	6	125	64	253
0.5 µM	0	11	49	9	181	250
T619Q-HA						
0 µM	27	3	18	126	98	272
0.5 µM	22	19	55	117	35	248

Experiment 1

PbGFP-Luc [TSP]	Gliding # of sporozoites	Drifting # of sporozoites	Waving # of sporozoites	Adherent # of sporozoites	Complex # of sporozoites	Total observed
0 µM	134	35	10	3	67	249
0.5 µM	83	55	18	11	39	206
2 µM	2	94	4	5	1	106

Experiment 2

PbGFP-Luc						
0 µM	104	15	26	14	37	196
0.5 µM	65	41	40	5	34	185
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	PKG-HA	T619Q-HA
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Number of LS, 48h p.i (mean ± SD)

	PKG-HA	T619Q-HA
Experiment 1 (n=4)	792 ± 18	332 ± 19
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Supplementary Table 2

A) % invaded sporozoites (mean ± SD)

	Control	CDPK4 cKO
Experiment 1 (n=4)	49 ± 7	17 ± 1
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Experiment 3 (n=4)	56 ± 9	25 ± 0.4
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Number of merosomes, 65-72 h p.i (mean ± SD)

	Control	CDPK4 cKO
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	Control	CDPK4 cKO
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Experiment 1	n = 5	n = 4
Day 4	3.20E-03 ± 2.21E-03	7.25E-04 ± 7.25E-04
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C) Effect of Compound 1294 on sporozoite motility

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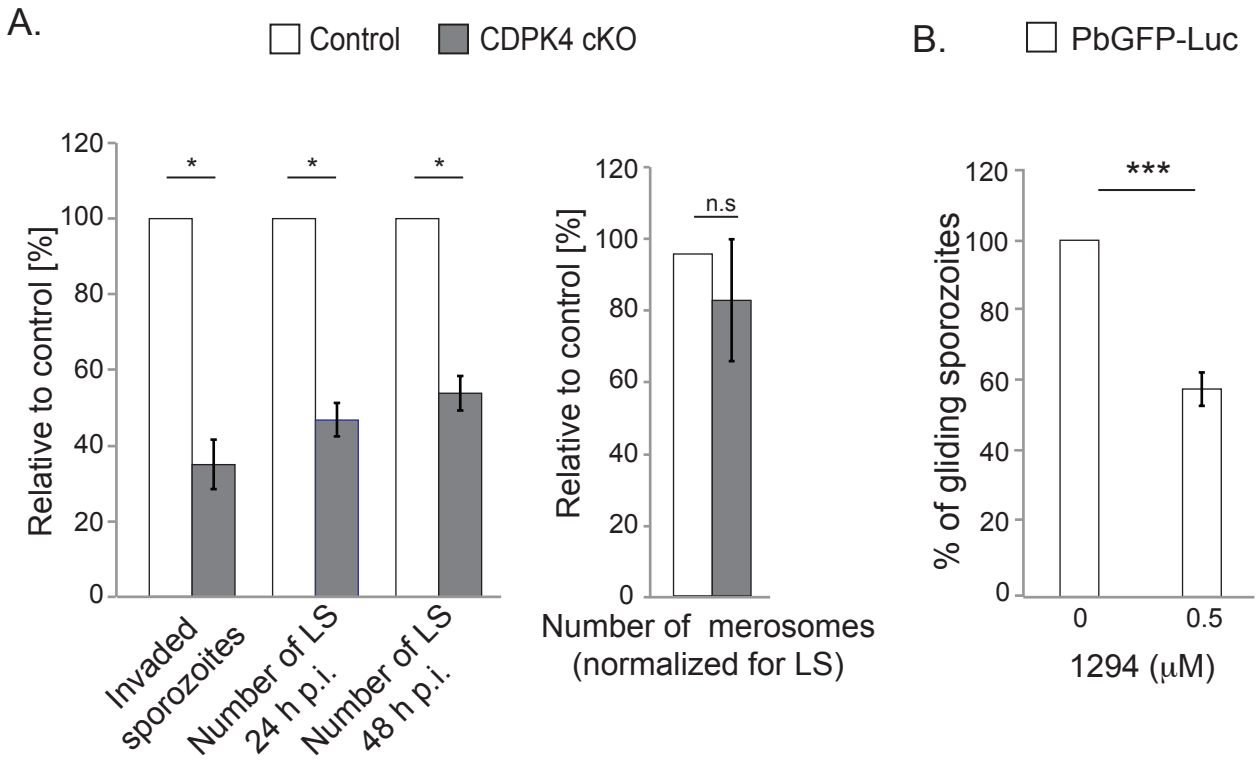
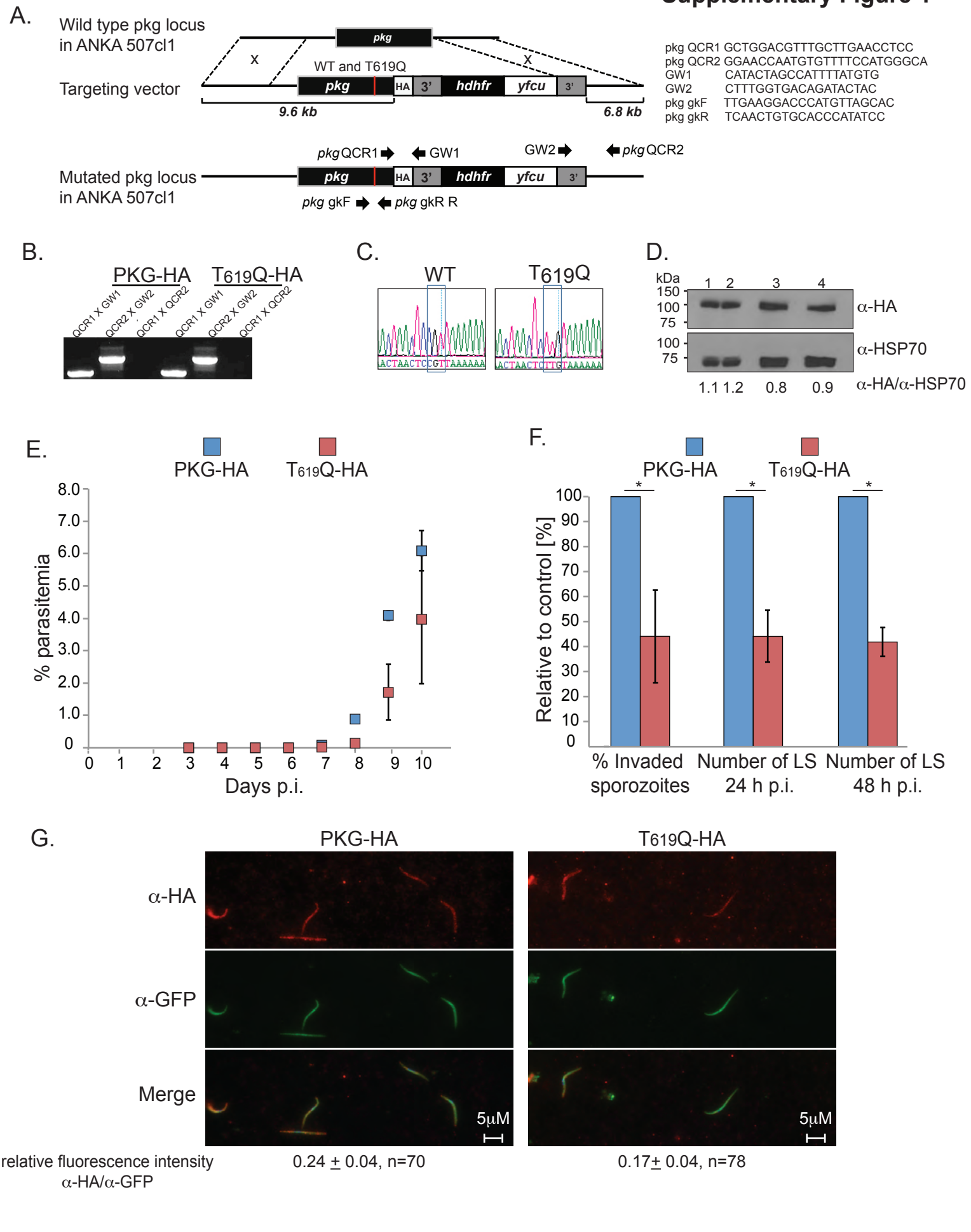
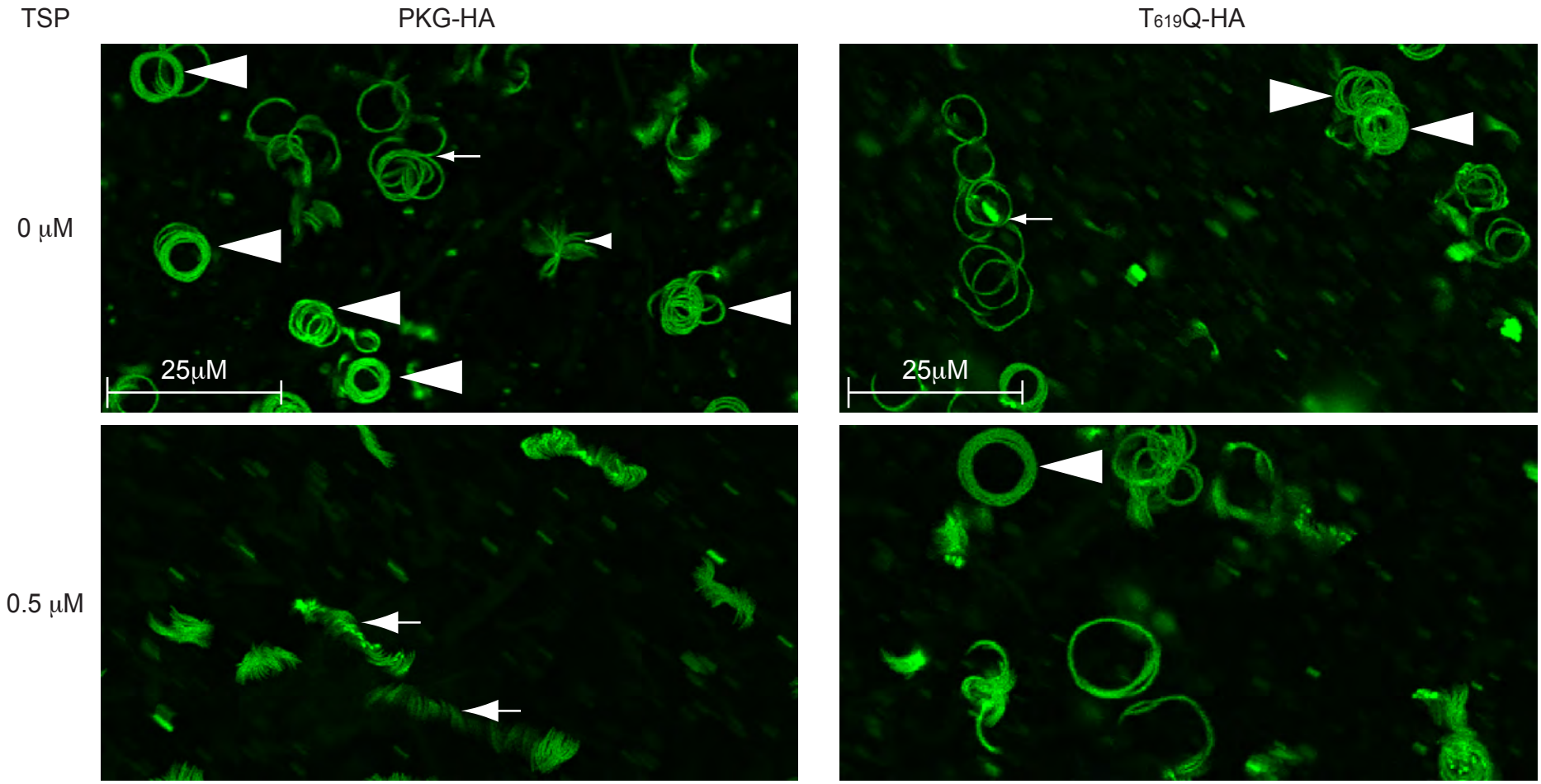
Figure 4


A.
Control
CDPK4 cKO
Relative to control [%]
120
100
80
60
40
20
0
*
*
*
Invaded sporozoites
Number of LS 24 h p.i.
Number of LS 48 h p.i.
n.s
Number of merosomes (normalized for LS)
B.
PbGFP-Luc
% of gliding sporozoites
***
0
0.5
1294 (μM)

Supplementary Figure 1



Supplementary Figure 2



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Experiment 3						
0 µM	36	4	13	68	8	129
0.5µM	18	1	27	68	33	147