Epistasis studies reveal redundancy among calcium-dependent protein kinases in motility and invasion of malaria parasites

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In malaria parasites, evolution of parasitism has been linked to functional optimisation. Despite this optimisation, most members of a calcium-dependent protein kinase (CDPK) family show genetic redundancy during erythrocytic proliferation. To identify relationships between phospho-signalling pathways, we here screen 294 genetic interactions among protein kinases in Plasmodium berghei. This reveals a synthetic negative interaction between a hypomorphic allele of the protein kinase G (PKG) and CDPK4 to control erythrocyte invasion which is conserved in P. falciparum. CDPK4 becomes critical when PKG-dependent calcium signals are attenuated to phosphorylate proteins important for the stability of the inner membrane complex, which serves as an anchor for the acto-myosin motor required for motility and invasion. Finally, we show that multiple kinases functionally complement CDPK4 during erythrocytic proliferation and transmission to the mosquito. This study reveals how CDPKs are wired within a stage-transcending signalling network to control motility and host cell invasion in malaria parasites.
Malaria is an infectious disease caused by mosquito-borne parasites of the genus *Plasmodium*. Protein kinases and phosphatases play important roles in regulating parasite development throughout their lifecycle. Gene knockout screens have revealed dozens of kinases that could not be disrupted in the asexual blood stages of either the human parasite, *P. falciparum*, or *P. berghei*, a model parasite which infects rodents. Other protein kinase genes can be readily disrupted in asexual blood stages, although their protein or transcript is expressed during these stages. This raises the possibility that despite the high degree of functional optimisation in the *Plasmodium* genome, some kinase functions may be masked, for instance, by compensatory roles of structurally related enzymes from the same family. Candidates include members of the CDPK family, mitogen-activated protein (MAP) kinases or tyrosine kinase-like (TKL) enzymes, as illustrated by the overexpression of the second MAPK (Pfmap-2) present in the *P. falciparum* kinome in parasites lacking the Pfmap-1 kinase. Functional roles of redundant genes can be revealed by genetic interaction studies where the combination of mutations in two or more genes generates unexpected phenotypes. Redundancy in signalling networks is common and has been demonstrated with great detail in yeast through genetic interaction screens. In search of functional links between protein kinases during erythrocytic proliferation of *P. berghei*, we screened 294 double and triple mutants. This revealed an unexpected negative interaction between calcium-dependent protein kinase 4 (CDPK4), a known regulator of cellular cycle progression during male sexual development but redundant in asexual stages, and modified alleles of protein kinase G (PKG), an essential kinase which controls key calcium signals across the lifecycle of malaria parasites.

During the erythrocytic phase of their lifecycle, parasites reproduce asexually producing merozoites that egress from the host red blood cell (RBC) and invade new RBCs within seconds. Regulation of egress and invasion of RBCs relies on multiple intracellular messengers, including calcium and cyclic guanosine monophosphate (cGMP), but the detailed architecture of the underpinning signalling networks remains poorly understood. *Plasmodium* components of cGMP signalling are much more diverged from mammalian enzymes and PKG is the only cGMP-dependent protein kinase in the parasite genome. This suggests that the threonine gatekeeper residue of PKG to a larger glutamine residue conferred reduced sensitivity to both inhibitors during egress and invasion, indicating that PKG is a primary target in these stages. Using this approach, we previously showed a major role for PKG in controlling calcium mobilisation from internal stores prior to RBC egress. This PKG-dependent calcium signal is likely transduced by CDPK5, which is also required for secretion of micronemal proteins essential for egress and invasion in *P. falciparum*. Another CDPK expressed in merozoites, CDPK4, was shown to be phosphorylated in a PKG-dependent manner and is involved in merozoite invasion.

PKG was further shown to regulate other calcium signals during mosquito transmission. Following ingestion by a mosquito, PKG mediates calcium mobilisation in the early steps of gametogenesis, after which CDPK4 and CDPK1 are required to complete gametogenesis. A similar functional relationship between PKG and CDPK4 might be conserved for the invasion of hepatocytes by sporozoites where both kinases are important for efficient gliding motility. PKG activity is also necessary to maintain high cellular calcium levels in ookinetes where calcium activates CDPK3, a kinase that supports efficient gliding and invasion of the epithelium of the mosquito midgut. Interestingly, a gliding phenotype can be restored in the absence of both CDPK3 and the GMP-specific phosphodiesterase delta (PDEδ). In this case, the lack of PDEδ over-activates PKG suggesting that, at least, one other unidentified calcium effector is involved in the regulation of ookinet motility. This apparent re-wiring suggests that a network of calcium effectors responds to PKG-mediated calcium signals.

Here, we have identified that in merozoites and ookinetes, PKG-dependent calcium signals activate multiple CDPKs, including CDPK1 and CDPK4, which show specific and complementary functions to sustain efficient gliding motility and invasion. These functions are likely mediated by the phosphorylation of multiple proteins required for the formation and the stability of the inner membrane complex (IMC). We also show that in ookinetes, PKG-dependent calcium signals are additionally effected by CDPK3 to control secretion of micronemal proteins. Altogether, this indicates that distinct CDPKs decode PKG-dependent calcium signals to coordinate microneme secretion with acto-myosin motor activity.

**Results**

A genetic screen reveals an interaction between *cdpk4* and *pkg*. To search for genetic interactions between *P. berghei* protein kinase genes, parasites from a panel of mutant clones lacking a specific kinase were negatively selected for loss of the selection marker and then transfected with a pool of barcoded gene knockout (KO) vectors to inactivate another kinase in the same background (Fig. 1a). The competitive growth rate of each mutant within the pool was measured during days 4–8 post infection by barcode sequencing. For the background lines, we focussed on the CDPK family and on the two atypical MAP kinases (Supplementary Data 1 and Supplementary Fig. 1A–D). In preliminary experiments, we found that a double mutant of *map1* and *map2* showed normal asexual growth (Supplementary Fig. 1A), and the double KO mutant was included in the screen as a single recipient background to identify interactors of either gene. Due to the essential role for PKG in calcium mobilisation upstream of CDPKs, we also included the inhibitor-resistant PKG*Te190->3xHA* line and its inhibitor-sensitive control, PKG*3xHA*. The library of KO vectors was comprised of 37 targeting vectors for protein kinases and 6 characterised vectors targeting unrelated genes for use as references (Supplementary Data 1 and ref. 4).

The screen examined 294 pairwise or 3-way combinations of mutant kinase alleles, the vast majority of which showed no evidence of interaction (Fig. 1b and Supplementary Data 2). Among these 294 combinations, 98 interaction tests involved one of the 14 vectors targeting genes likely essential in wild type. None of these became viable in any of the mutant backgrounds, i.e., there were no instances of strong positive interactions. No cases of synthetic lethality were observed. Most notably, no negative interactions among CDPKs were detected, as none of the pairwise deletions of CDPKs showed reduced growth beyond that of the single mutants (Supplementary Fig. 2). However, two negative interactions were both statistically significant and of sufficient magnitude to warrant further investigation (Fig. 1b).

Both involved disruption of *cdpk4* in the presence of a modified allele of *pkg*, with the drug-resistant *pkg*Te190->3xHA allele (*ε = −0.44, p value = 0.009), two-tailed t-test) imparting a more pronounced fitness cost to *cdpk4* deletion, when compared with the drug-sensitive *pkg*-3xHA background (ε = −0.30, p value = 0.02, two-tailed t-test). These data suggest that either the epitope
tag or the generic 3′-untranslated region (UTR) following the tag affected the function of PKG in a way that sensitised parasites to the deletion of cpdk4, an effect that was exacerbated by the T619Q substitution in the PKG active site.

To verify the negative interaction between pkg and cpdk4, a cpdk4-KO/pkgT619Q-3xHA clonal line was generated and found to have severely reduced growth (Fig. 1c) if compared to either of the single mutants (Supplementary Fig. 3A–B). Cis-complementing this line by re-inserting 3xHA epitope tagged wild-type alleles of either cpdk4 or pkg in their respective genomic loci (Supplementary Fig. 3C–D) restored parasite growth only partially (Supplementary Fig. 3E), possibly because the generic 3′UTR or the epitope tag used to monitor successful complementation creates hypomorphic alleles for both CDPK4 and PKG. Confirming this, cis-complementing the double mutant with non-epitope tagged wild-type alleles of either cpdk4 or pkg (Supplementary Fig. 3F–G) restores erythrocytic growth almost to wild-type levels, validating the interaction between PKG and

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\text{Interaction coefficient of genes } x y = \frac{w_{xy}}{w_x w_y} \\
\text{Relative growth rate of mutant } x = \frac{w_x}{w_{\text{wild-type}}}
\]
CDPK4 (Fig. 1d). A similar fitness cost of the generic 3'UTR and the epitope haemagglutinin (HA) tag is also observed during the sexual development of the parasite (Supplementary Note 1 and Supplementary Fig. 4A–G).

The pkg/cdpk4 interaction is required for merozoite invasion. Schizonts are mature erythrocytic stages that following asexual replication contain merozoites which, upon RBC egress, are able to infect new RBCs. Schizonts of the cdpk4-KO/pkgT619Q-3xHA line contain the same number of merozoites as the wild type (Fig. 1e), but when injected intravenously into mice, they show a reduced capacity to transform into ring stages while no significant amount of circulating schizonts is observed (Fig. 1f), suggesting that pkg and cdpk4 genetically interact late in the growth cycle to control either the final stage of schizont maturation or invasion of new erythrocytes.

An ultrastructural analysis of cdpk4-KO/pkgT619Q-3xHA schizonts by transmission electron microscopy (TEM) revealed that a significantly higher number of mutant merozoites show a discontinuous IMC with gaps, while single mutants did not (Fig. 1g, h). The IMC is made up of alveoli beneath the plasma membrane and is important to maintain the stability of the parasite. Importantly, it is acting as an anchor for a protein complex known as the glideosome, which is crucial for host cell invasion.

The pkg/cdpk4 interaction is conserved in P. falciparum merozoites. To further investigate the interaction between PKG and CDPK4, we then focussed on egress and invasion in P. falciparum. We first aimed at generating a PPKG T618Q/CDPK4-KO parasite line using CRISPR-Cas9. Attempts to disrupt the CDPK4 catalytic domain were only successful on a wild-type background (Supplementary Fig. 5A) and not in an existing line expressing the T618Q allele of PKG25, suggesting that the observed interaction in P. berghei may translate to P. falciparum, but is likely synthetic lethal and thus not tractable by genetic deletion of cdpk4.

We reasoned, more conclusive evidence could be obtained with PKG inhibitors that may mimic the effect of the T618Q substitution in the CDPK4-KO background. First, we used the imidazopyridine C2, which was shown to inhibit PKG both in vitro and in vivo25,26. Synchronised mature schizonts were treated for 3 h with C2 and the number of ring and schizont parasites were then determined by flow cytometry (Supplementary Fig. 5B–C). A control line expressing the C2-resistant PPKG T618Q allele is not affected at 4 μM, confirming PKG is the main target of C2 to block merozoite egress and invasion25. Unexpectedly, we observed a higher EC_{50} of 0.8 μM for ring formation in the CDPK4-KO line compared with 0.4 μM for the 3D7 control line despite a similar half-maximal effective concentration (EC_{50}) for schizont rupture of 0.8 μM for both lines (Fig. 2a). This indicates that for the same number of ruptured schizonts, the CDPK4-KO line gives rise to more ring parasites than the wild type in the presence of C2. This suggests that in the wild type, C2 has a second target important for invasion that is absent or not active in the CDPK4-KO line.

C2 has previously been suggested to inhibit both PPKG and PICDPK4 during male gametogenesis24. This dual specificity could be explained because both kinases share a relatively small gatekeeper residue (Thr and Ser, respectively) in the adenosine triphosphate (ATP) binding pocket required for inhibitor binding. The higher sensitivity to C2 of the 3D7 parental line for ring formation could therefore be due to a dual effect of C2 on both PKG and CDPK4. This possibility is supported by two lines of evidence. First, we confirm CDPK4 as an additional target for C2 in P. falciparum merozoites, by complementing the PICDPK4-KO line with episomally expressed PbCDPK4-2xmyc or PbCDPK4 S147M-2xmyc, respectively (Supplementary Fig. S5D). Complementation with PbCDPK4-2xmyc lowers ring formation in the presence of C2, while complementation with the PbCDPK4 S147M-2xmyc allele does not (Fig. 2b). The S147M gatekeeper mutation in CDPK4 is known to confer resistance to another kinase inhibitor exploiting the small gatekeeper residue in the ATP binding pocket of CDPK412,19. It is reasonable to assume that the same mutation would also block binding of C2, which in PKG is known to exploit the small gatekeeper of this kinase. Similarly, we observed that C2 also targets both PKG and CDPK4 during P. berghei gametogenesis, further substantiating the dual specificity of C2 across the malaria lifecycle (Supplementary Note 1 and Supplementary Fig. 4B–F). Secondly, we investigated whether an unrelated inhibitor of PKG that does not target CDPK4 (Supplementary Note 1, Supplementary Fig. 4C and ref. 25), the imidazopyridrazine Compound A, would show the same difference in EC_{50} for ring formation as the CDPK4-KO line. As opposed to C2, Compound A shows the same EC_{50} for schizont rupture and ring formation in both 3D7 and CDPK4-KO lines (Fig. 2c). This further points to subtle off-target effects on CDPK4 of C2 during merozoite invasion.

Altogether, these data indicate that C2 affects merozoite invasion by targeting both PKG and CDPK4 in the wild-type background, while in the CDPK4-KO line, the absence of CDPK4 is possibly compensated by one or multiple unknown C2-insensitive kinases, leading to a higher resistance to C2 for merozoite invasion.

CDPK4 function is revealed by attenuated calcium signals. Since in gametocytes CDPK4 is a known effector of a PKG-dependent calcium signal, we hypothesised that PPKG T618Q represents a hypomorphic allele encoding a kinase that signals less effectively. Consistent with this idea, the T618Q mutation in recombinant PPKG raises the affinity constant for ATP from 19.59 μM to 87.60 μM in vitro (Fig. 2d). In schizonts of the
PPKGT618Q line, this has no effect on baseline Ca\textsuperscript{2+} levels in asexual blood stages (Fig. 2e). Raising cellular cGMP levels through phosphodiesterase inhibitors leads to a PKG-dependent increase in cytosolic Ca\textsuperscript{2+} in schizonts\textsuperscript{13}. This effect is significantly reduced in parasites expressing the PPKGT618Q allele (Fig. 2f and Supplementary Fig. 6A–C), although total cellular calcium available for release by an ionophore is unchanged. Similarly, we found that \textit{P. berghei} parasites expressing the PKG\textsuperscript{618Q} allele show reduced intracellular Ca\textsuperscript{2+} mobilisation during early gametogenesis (Supplementary Note 1 and Supplementary Fig. 4G). Altogether, this suggests that the increased requirement for CDPK4 in the PKG gatekeeper mutant lines results from weaker PKG-dependent calcium signals.

CDPK4 is at the interface between the IMC and the glideosome. While calcium levels can account for the functional interaction between \textit{pkg} and \textit{cdpk4} mutations, they do not explain how CDPK4 can affect RBC invasion by the merozoite. To address this question, we tagged CDPK4 in \textit{PbANKA} 2.33, a line unable to produce gametocytes\textsuperscript{28}, and confirmed its expression in erythrocytic schizonts (Fig. 3a and Supplementary Fig. 7A). Immunofluorescence localisation shows a signal excluded from the nucleus with a slight enrichment at the merozoite periphery (Fig. 3a). In CDPK4-3xHA immunoprecipitates following cross-linking, 19 proteins are enriched over wild-type controls (Supplementary Data 3), including GAP40, MyoA and GAC, three proteins essential for the IMC biogenesis or gliding motility\textsuperscript{29,30}. In addition to MyoA, CDPK4 also immunoprecipitates an uncharacterised \textit{Plasmodium}-specific myosin, MyoE (PBANKA_011220). Altogether, this further suggests that the redundant role of CDPK4 observed in invasion could be linked to the regulation of the molecular machinery that forms the IMC or provides the force for invasion.

Since we previously found GAP40\textsuperscript{S448/S449} among a number of sites phosphorylated by an analogue-sensitive CDPK4\textsuperscript{S147G} (ref. \textsuperscript{12}), we chose to first validate this putative interaction. Endogenously tagged GAP40 (Supplementary Fig. 7B) localises to the parasite periphery (Fig. 3a). Among the 20 proteins co-immunoprecipitated with GAP40-3xHA is CDPK4, in addition to known IMC and glideosome components (Fig. 3b and Supplementary Data 3). Since gap40 is likely essential for asexual growth\textsuperscript{12}, we chose to examine specifically the relevance of GAP40\textsuperscript{S448/S449} phosphorylation by substituting both serines with alanine residues. This does not affect parasite growth or integrity of the IMC in either the wild-type nor the PKG\textsuperscript{619Q}-3xHA/CDPK4-KO background (Fig. 3c, d). The absence of an obvious phenotype for these substitutions could be explained either by the fact that phosphorylation of residues 448 and 449 of GAP40 is functionally not important or by the high levels of functional plasticity observed for the phosphorylation of glideosome components in apicomplexan parasites\textsuperscript{31}.

We were intrigued to find that GAP40-3xHA also co-immunoprecipitates another member of the CDPK family involved in
Fig. 3 CDPK4 co-immunoprecipitates with components of the glideosome and the IMC. a Western blots and immunofluorescence analysis of schizonts expressing endogenously 3xHA tagged cdpk4, gap40, cdpk1 and soc6 alleles. Scale bars are 1μm. b Protein interactions between IMC or glideosome proteins identified from GAP40-3xHA, SOC6-3xHA, MyoE-3xHA, CDPK4-3xHA and CDPK1-3xHA immunoprecipitates. Thick bars indicate that the interaction was identified from both immunoprecipitates. Blue and green filled circles denote the number of residues phosphorylated by CDPK4 ex vivo and by recombinant CDPK1 in vitro, respectively. The colour of the circle around the protein name indicates the requirement of its encoding gene for growth in asexual blood stages: red is essential, green is redundant; black denotes that the gene essentiality was not tested. c Effect of gap40 mutagenesis on the growth of asexual blood stage parasites (error bars show standard deviations from 2 independent infections). d Electron microscopy analysis of mature WT and GAP40S448/449A-3xHA schizonts (error bars show standard deviations from the mean; duplicates; two-tailed t-test; nWT = 140, nGAP40S448/449A-3xHA = 132)

merozoite invasion, CDPK1, and again, MyoE. Epitope tagged CDPK1 and MyoE (Supplementary Fig. 7C–D) are enriched at the cell periphery (Fig. 3a and Supplementary Fig. 7D), and both proteins co-immunoprecipitate multiple glideosome or IMC components (Fig. 3b and Supplementary Data 3). Collectively, these results suggest MyoE may act as an alternative myosin of the motor complex and that both CDPK1 and CDPK4 are at the interface between the glideosome and the IMC. This may contribute to the genetic buffering observed when deleting these enzymes individually, although the initial screen found no strong evidence for a genetic interaction between them (Supplementary Data 1).

CDPK4 phosphorylates a protein involved in IMC stability. Immunoprecipitation of GAP40 or MyoE recovers multiple peptides from a protein of unknown function which, like GAP40 itself, was one of a small number of hits that emerged from our recent biochemical screen for substrates of CDPK4 (SOC proteins) in parasite lysates. This protein, SOC6 (PBANKA_070770), has since been shown to interact with the IMC protein Phil1 in P. berghei schizonts and with MyoA in P. falciparum schizonts, and may thus provide a molecular link between CDPK4 and invasion. SOC6 is characterised by a C-terminal stretch of 106 amino acids that are relatively conserved across the tyrosine orthologues of different malaria parasites (Supplementary Fig. 8A), but lacks obvious homologues in other apicomplexan genomes. SOC6 is further characterised by 4 to 15 tandem amino acid repeats that show sequence and position variability across species (Supplementary Fig. 8B). In P. berghei, the serine residue that CDPK4 phosphorylates in vitro lies in one such repeat of 54 amino acids (Supplementary Fig. 8A), which has prevented us from mutagenising specifically the phosphosite.

Endogenously tagged SOC6-3xHA (Supplementary Fig. 7E) localises to the cell periphery of merozoites (Fig. 3a) and immunoprecipitates peptides from multiple IMC, glideosome-associated proteins and glideosome proteins (Fig. 3b and Supplementary Data 3). Altogether, this indicates that SOC6 is also at the interface between the IMC and the glideosome. A SOC6-KO line shows a significant growth defect compared with wild type (Fig. 4a). While segmented SOC6-KO schizonts display the same number of merozoites as wild type (Fig. 4b), they show a reduced capacity to transform into ring stage parasites, while no accumulation of circulating SOC6-KO schizonts is observed.
This indicates SOC6 is important either at the final stage of schizont maturation or to invade new RBCs. TEM of purified SOC6-KO schizonts reveals a discontinuous IMC as observed for PKG7619Q-3xHA/CDPK4-KO transgenic (Fig. 4d and Supplementary Fig. 8C), suggesting that SOC6 is important for the formation or the stability of the IMC in merozoites.

To investigate the function of SOC6 further, we turned to the ookinete stage, which in P. berghei offers a tractable model to study the molecular motor that powers gliding motility. Ookinetes emerge from the zygote that forms after fertilisation of macrogametes by microgametes in the mosquito blood meal. Male gamete formation does not require SOC634, but the SOC6-KO nevertheless fails to form typical banana-shaped ookinetes (Fig. 4e). Again, TEM of SOC6-KO cells reveals either a discontinuous IMC or the complete absence of an IMC below the plasma membrane (Fig. 4f, g), suggesting that SOC6 plays a conserved role to control the IMC formation or stability at multiple stages of the malaria lifecycle.

CDPK4 and 1 functionally interact to control invasion and motility. The presence of CDPK1 at the pellicle of P. berghei is consistent with its proposed function in phosphorylating a number of proteins possibly involved in motility and invasion35,36. Furthermore, there is evidence that CDPK1 functionally interacts with PKG in P. falciparum merozoites37 and is important for erythrocyte invasion38. On the other hand, P. falciparum asexual blood stages can adapt to the loss of CDPK121.
and in *P. berghei*, neither CDPK1-KO nor the double CDPK1-KO/CDPK4-KO nor PKG T619Q-3xHA/CDPK1-KO parasites show a significant growth defect (Supplementary Data 1). However, we were not able to generate a triple PKGT619Q-3xHA/CDPK4-KO/CDPK1-KO mutant, while CDPK3 and CDPK6 could be readily knocked out in the PKGT619Q-3xHA/CDPK4-KO background (Fig. 5a). These results suggest that CDPK1 may partially complement the absence of CDPK4 and vice versa, and that another unidentiﬁed kinase is involved in this calcium signalling pathway downstream of PKG. In an attempt to identify other protein kinases that could compensate for loss of CDPK4 and CDPK1, we immunoprecipitated GAP40-3xHA or MyoE-3xHA in the CDPK4-KO background, but no significant differences could be detected (Supplementary Fig. 7F).

PKG, CDPK4 and CDPK1 are abundantly expressed in ookinetes, and gliding requires PKG, since addition of 0.5 µM C2 almost completely blocks wild-type motility, while it has no effect on the C2-resistant PKGT619Q-3xHA mutant (Fig. 5b). We next asked whether PKG-dependent gliding could be used to reveal roles for CDPK4 and CDPK1. As PbCDPK1 is essential for ookinetes to develop, we used a PbCDPK1-AID-HA line in which CDPK1 is inducibly degraded with the help of an auxin-dependent degron fused to the C terminus of the protein kinase (Fig. 5c and ref. 39). PbCDPK1-AID-HA ookinetes show normal...
gliding speed irrespective of auxin addition (Fig. 5d). Similarly, a CDPK4 selective inhibitor 1294\(^\text{[12,19]}\) does not affect motility of PbCDPK1-AID-HA ookinetes at 1 \(\mu\)M in the absence of auxin. These data indicate that neither CDPK4 nor CDPK1 is individually required for gliding. However, when CDPK1-AID-HA is destabilised with auxin, the CDPK4 inhibitor significantly decreases ookinete speed, indicating that both enzymes control ookinete gliding and functionally complement each other (Fig. 5d).

In *P. falciparum* schizonts and *P. berghei* sporozoites, PKG controls microneme secretion\(^\text{[10]}\), a process that is also critical to sustain gliding in ookinetes. C2 blocks the secretion of the ookinete microneme protein CelTOS-3xHA (Supplementary Fig. 9A) into the culture supernatant, specifically in the inhibitor-sensitive line (Fig. 5e), indicating that signalling through PKG is required for microneme secretion also in ookinetes. However, depletion of CDPK1 and chemical inhibition of CDPK4 does not affect secretion of CelTOS-3xHA either individually, or in combination (Fig. 5f). In marked contrast, deletion of CDPK3, an ookinete-specific CDPK needed for optimal gliding (Fig. 5g and refs. \(^\text{23,41}\)), does reduce secretion of CelTOS-3xHA (Fig. 5h and Supplementary Fig. 9B, C). Complementation of *cdpk3* deletion ascertained that this effect was due to the absence of *cdpk3* expression (Supplementary Fig. 9D-E). Furthermore, CDPK3 does not appear to interact functionally with CDPK4, since addition of 1294 does not decrease motility further in the CDPK3-KO (Fig. 5g). Altogether, this suggests that the main function of CDPK3 is to control microneme secretion downstream of PKG but independently of CDPK4, while CDPK1 and CDPK4 perform complementary functions in supporting efficient gliding.

**Discussion**

Genetic interactions occur when mutations in two or more genes combine to generate an unexpected phenotype. Comprehensive interaction studies are a major undertaking owing to the combinatorial complexity of generating double mutants for each pairwise interaction tested, particularly in non-model organisms such as malaria parasites. A recent development in the genetic manipulation of *P. berghei* now enables large-scale reverse genetic studies in this species\(^\text{41}\). Using this approach, we describe the first genetic interaction screen in a malaria parasite. We examined 294 pairwise or 3-way combinations of mutant kinase alleles, the vast majority of which showed no evidence of interaction. We were only able to detect two negative interactions both involving disruption of the otherwise redundant *cdpk4* gene in the presence of hypomorphic alleles of *pkg*. This number appears to be low when compared to yeast, where around 1 million interactions were identified out of 23 million double mutants\(^\text{42}\). As signalling cascades are more frequently associated with functional redundancy, as exemplified by the mammalian MAP kinases, nuclear factor-\(\kappa\)B or Wnt pathways, these results suggest a possible reduction of genetic interaction networks in malaria parasites possibly linked to functional optimisation during evolution of parasitism\(^\text{8}\). However, our analysis mainly interrogated a subset of redundant kinases. As essential genes in yeast display five times as many interactions as non-essential genes\(^\text{12}\), it is difficult to draw definitive conclusions regarding the extent of gene interaction networks in malaria parasites.

Genetic interaction networks highlight mechanistic connections between genes and their corresponding pathways. As PKG was shown to regulate both merozoite egress and invasion, the negative interaction with CDPK4 strongly suggested a role for the latter in one of the two processes. Such a role for CDPK4 was unexpected as its known functions were to control unrelated processes during cell cycle transitions in microgametocytes\(^\text{12}\). Here, we reveal that CDPK4 is also important for the stability of the IMC, which serves as an anchor for the acto-myosin motor that is essential for *P. falciparum* invasion but not egress\(^\text{43}\). This functional interaction between PKG and CDPK4 to control gliding may be conserved in sporozoites where both enzymes support efficient gliding\(^\text{26}\). Signals transduced by CDPK4 are possibly mediated by phosphorylation of at least GAP40 and SOC6, which are both important for the biogenesis\(^\text{44}\) and the stability of the IMC. SOC6 co-immunoprecipitated with Phil1 that was recently proposed to be important for IMC plate expansion\(^\text{32}\) and it is possible that SOC6 plays a similar role. It is important to note that the molecular role of CDPK4-mediated phosphorylation remains unresolved as the SOC6-KO phenotype may be unrelated to its phosphorylation status. Further work will be necessary to comprehensively identify CDPK4 substrates in merozoites and dissect the molecular role of CDPK4-dependent phosphorylation events.

The impact of protein phosphorylation in controlling motility and invasion remains poorly understood in malaria parasites. A limited number of protein kinases have been shown to control gliding motility or invasion in *Plasmodium*. The strict requirement for PKG in both processes is probably pleiotropic due to its critical role in calcium regulation upstream of protein secretion and possibly regulation of the acto-myosin motor itself, as suggested by this study. The role of CDPKs downstream of PKG-dependent signals in motility and invasion also remains elusive. PICDPK1 was proposed to directly regulate the glideosome as the recombinant enzyme phosphorylates PITMIP and PMy0A in vitro\(^\text{35}\). Phosphorylation of multiple IMC proteins was also shown to depend on CDPK1\(^\text{36}\), further suggesting that CDPK1 participates in the assembly or stability of the IMC. The role of CDPK1 in invasion was also possibly linked to microneme
secretion. However, secretion of AMA1, a protein essential for merozoite invasion, was not CDPK1 dependent. However, the exact contribution of CDPK1 to invasion remains unclear as merozoites lacking the kinase remain invasive possibly due to the re-wiring of underlying signalling networks in its absence. Interestingly, CDPK5 was also shown to be essential for the secretion of micronemal proteins involved in merozoite invasion. This requirement was shown to become redundant when PKG is over-activated by a PDE inhibitor, suggesting that CDPK5 is part of the CDPK-dependent network downstream of PKG and may account for the redundancy of both CDPK4 and CDPK1 in merozoite and ookinetes. The role of CDPK3 in ookinete gliding was unknown and we show here that it controls microneme secretion. It is important to note that the essential protein kinase A (PKA) was proposed to be important for invasion in apicomplexan parasites by interplaying with cGMP- and calcium-dependent signalling. Our genetic screen did not allow to study negative interactions with or among essential genes and it is highly likely that the cGMP/Ca2+-dependent signalling network we describe here includes multiple other kinases including PKA. More work will be required to fully appreciate the exact architecture and plasticity of this signalling network. Nevertheless, this work suggests that in ookinetes and merozoites, PKG-dependent calcium signals are decoded by distinct but overlapping CDPK networks to coordinate microneme secretion and the acto-myosin motor with stage-transcending components, such as CDPK4, CDPK1, possibly CDPK5, and more stage-specific regulators such as CDPK3 (Fig. 6).

CDPKs are encoded by a large multigene family that is present in plants, protists, oomycetes and green algae, but is not found in animals and fungi. It was proposed that protist and plant CDPK diversification into multiple gene family groups are independent of each other. Despite gene family expansion, CDPK gene sequences appear to be highly conserved, which could explain frequently observed functional redundancy among these enzymes. For example, whereas biochemical approaches show distinct molecular functions of CDPKs in Arabidopsis, gene disruption phenotypes of CDPKs have only been rarely reported. This raises the intriguing question of why this gene family is amplified and diversified. It is clear that Plasmodium CDPKs evolved unique cellular functions. However, our results indicate that they also retained functional redundancy, presumably to reach a threshold of global calcium-dependent kinase activity. It is interesting to note that CDPK1 did not functionally interact with hypomorphic alleles of PKG, suggesting it may require lower calcium levels than CDPK4. Interestingly, this CDPK network downstream of PKG-dependent calcium signals is conserved to transduce distinct signals in merozoites, gametocytes and ookinetes. These features may allow for a balance between evolvability to adapt to species- or stage-specific requirements and robustness of calcium-dependent signalling. For example, the requirement for CDPKs is slightly different between P. berghei and P. falciparum for gametogenesis or merozoite invasion, suggesting that if the same network is involved in both processes, the wiring might be slightly different due to species-specific factors. CDPKs that have a single function at a specific stage or unrelated developmental stages may also constrain the evolvability of CDPK networks. A similar situation is observed in the related apicomplexan parasite Toxoplasma gondii, where CDPKs show a high degree of redundancy to control cellular processes including parasite egress and invasion. For example, microneme secretion universally depends on TgCDPK1, but only exhibits TgCDPK3 dependence when triggered by certain stimuli. A better understanding of each kinase molecular function among different parasites and stages may further reveal how CDPK networks evolved for specific species or genus requirements within apicomplexan parasites.

By screening for genetic interactions among a subset of protein kinases, we have revealed an unexpected role for CDPK4 downstream of PKG-mediated calcium signals. To better map the signalling networks downstream of PKG-mediated signals across the lifecycle, it will be interesting to screen for interactions between CDPKs under conditions where PKG is over-activated, either by chemical inhibition or deletion of cGMP-specific phosphodiesterases. Similar analyses will also prove extremely valuable to gain a more comprehensive view of phospho-signalling pathways by, for example, screening for genetic interaction among kinases and phosphatases or using conditional approaches to study genetic interactions with essential genes.

**Methods**

**Preparation of targeting vectors.** 3xHA tagging, knockout and allelic replacement constructs in P. berghei were generated using phage recombineering in Escherichia coli tryptic soy agar (TSA) bacterial strain with PlasmoGEM vectors (http://plasmogem.sanger.ac.uk/). Vectors available in the PlasmoGEM repository are listed in Supplementary Data 1. For final targeting vectors not available in the PlasmoGEM repository, generation targeting constructs were performed using sequential recombineering and gateway steps. A list of oligonucleotides used in this study is available in Supplementary Data 4. For each gene of interest (goi), the Zeocin-resistance/Phe-sensitivity cassette was introduced using oligonucleotides goi HA-F x goi HA-R for 3xHA tagging. Substitution of the GAP45/148/153A residue was introduced using primer gap45/148/153A instead of gap40 HA-F. Mutations were confirmed by sequencing with primers gap40-OC1 and GW1. The modified library inserts were released from the plasmid backbone using NotI.

To generate a new gateway entry cassette allowing simultaneously to introduce an in-frame AID-HA tag and express the Tir1 protein in ookinetes (generation of a CDPK3-KO/CelTOS-AID-HA/Tir1 line), the gateway entry vector for AID-HA tagging from the PlasmoGEM resource was first linearised by KpnI. The osTIR1 gene, hsp70 promoter and p28 3′UTR were amplified from genomic DNA of P. berghei CDPK1-AID-HA line with oligonucleotides osTIR1 F x osTIR1 R, Phsp70 F x Phsp70 R and p28 3′UTR F x p28 3′UTR R. Linearised vectors and PCR amplicons were then assembled by Gibson Assembly to create the circular GW-AID-HA-Tir1 vector.

For the pICDPK4-KO construct, homologous regions were cloned into the pcpp1 plasmid. A first homology region mapping upstream of pfcdpk4 was amplified with the following primers:

**References:**

1. Nature Communications | DOI: 10.1038/s41467-018-06733-w | www.nature.com/naturecommunications
oligos PCDPK4 H1-R and PCDPK4 H1-R and ligated into SacII/AflII digested pc1. A second homology region mapping downstream of the region encoding CDPK4 catalytic domain was amplified using PCDPK4 H2-R and PCDPK4 H2-R and ligated into Ncol/ArifI digested pc1 to generate pc1-CDPK4-KO plasmid. The gRNA oligonucleotides PCDPK4 gRNA F and PCDPK4 gRNA R were ligated into pc1 digested with Ncol/Arai and Gibson assembled in the digested plasmid. To generate PPK4147M homozygous KO, we used the tdhfr gene of the pc1-CDPK4-KO digested plasmid by digesting the vector with AflII and HindIII. The bsd gene was amplified using BSdWarp F x BSdWarp R. The resulting amplicon was digested with AflII and HindIII cloned into the above vector. Sequence of the bsd gene was confirmed by Sanger sequencing using primers bsd seq1 and 2. For PCDPK4 F and cdpk4-S147M R and further Gibson assembled in the digested plasmid. The sequence of the cdpk4 gene was confirmed by Sanger sequencing using primers cdpk4-seq1F to cdpk4-seq4F and GW1.

Parasites and animals. P. berghei strain ANKA52 derived clone 2341 and derived transgenic lines were maintained in female CD1 outbred mice. The parasitaemia of infected animals was determined by methanol-fixed and Giemsa-stained thin blood smears. C57BL/6J mice were obtained from Charles River Laboratories and were specific-pathogen free and subjected to regular pathogen monitoring by sentinel screening. Mice were housed in individually ventilated cages furnished with a cardboard mouse house and Nestlet. Mice were maintained at 21 ± 2 °C under a 12 h light/dark cycle and given commercially prepared autoclaved rodent chow and water ad libitum. Female mice were used for experimentation at 6–10 weeks of age and were randomly selected for parasite infection. The investigator was blinded to the parasite group allocation for the intraocular injection of P. berghei schizonts. Animal experiments were conducted with the authorization numbers GE/82/15 and GE/201/17 according to the guidelines and regulations issued by the Swiss Federal Veterinary Office. The procedures were approved by the Swiss Federal Veterinary Office in accordance with National and European animal welfare guidelines. For the analysis of isolated transgenic parasites, the sample size was chosen to ensure a power of at least 80% using G*Power (http://www.gpower.hhu.de/). Statistical analyses were performed using GraphPad Prism 7.

P. berghei culture and transfection. Schizonts for transfection were purified from overnight cultures on a Histoderm cushion made up from 55% of a Histoderm stock and 45% phosphate-buffered saline (PBS). Purified parasites were harvested from the interface and centrifuged at 500 × g for 3 min. Two protocols have been used for parasite electrotransformation. In the first one used at the University of Geneva, cells were resuspended in 100 µL Amaza Basic parasite Nucleofector solution (Lonza), added to 10–20 µg of precipitated DNA re-suspended in 10 µL of H2O and electrotransfected using the Amaxa Nucleofector II (Amaxa) with a program of the Amaza Nucleofector II. Nucleofector delivered parasites were resuspended in 200 µL of fresh red blood cells and injected intraperitoneally into mice. Selection with 0.14 mg/mL pyrimethamine (Sigma) in drinking water (pH ~4.5) was initiated from day 1 post infection. In the second protocol used at the Sanger Institute, schizonts were re-suspended in 18 µL Amaza Primary cells Nucleofector solution (Lonza) and added to ~5 µg of precipitated DNA re-suspended in 5 µL of H2O. Cells were electroporated using the FI-115 program of the Amaza Nucleofector 4D and transfected parasites were directly injected intravenously into the tail vein of mice. Selection with 0.07 mg/mL pyrimethamine (Sigma) in drinking water (pH ~4.5) was initiated from day 1 post infection. Negative selection of parasites expressing YF9C (a bifunctional protein that combines yeast cytosine deaminase and uridyl phosphoribosyl transferase) was performed through the administration of 50 µg/mL 6-mercaptopurine via drinking water (pH ~4.5) was initiated from day 1 post infection. Parasite genomic DNA was phenol/chloroform extracted from these samples5. Growth rates of single, double and triple mutants were measured using the gene-specific 11 bp barcode present in each of Plasmodium species vectors, which are found in the PlasmoGEM database (http://plasmogem.sanger.ac.uk). Infections were sampled at the same time of each day between days 4 and 8 post infection. Parasite genomic DNA extracts and counted on an Illumina MiSeq. For that, ampiclon-based Illumina sequencing libraries were prepared using a nested PCR approach to yield 234 bp long amplicons containing sample-specific indexes that were pooled equimolarly in groups of 32. Growth rates of single, double or triple mutants were calculated using an algorithm developed in ref. 8. These data were used to calculate interaction coefficients using a subtractive model whereby the relative fitness of each single mutant (i.e., transfection performed on the wild-type background) was subtracted from the relative fitness measured for each parasite in single-transfection (or three-way test (i.e., transfection performed on each background combination with each single mutant backgrounds): Relative fitness of mutant x in wild-type background = W0x, Wx in background = y = Wxy, interaction coefficient genes x y = wy, where wy = Wxy – W0x, W0y.

Protein analysis. Figure source data for all western blots are shown in Supplementary Fig. 10. Co-immunoprecipitation of CDPK4-3xHA, CDPK1-3xHA, MyoE-3xHA, SOC6-3xHA, GAP40-3xHA protein complexes were performed with schizozoite fixed for 10 min with 1% formaldehyde, lysed in RIPA buffer and the supernatant was subjected to affinity purification with magnetic beads conjugated with monoclonal anti-HA antibody, clone 3F10 (Sigma-Aldrich, reference 000000011867431001). A WT control was included in parallel and proteins for which we recovered peptides in the WT control were not retained for further analysis. Magnetic beads were washed in 100 µL of 6 M urea in 50 mM Tris–HCL, pH 8.0, 5 mM ethylenediaminetetraacetic acid (EGTA) to block nonspecific interactions. Beads were washed in 50 mM in liquid chromatography–mass spectrometry (LC-MS) grade water were added and the reduction was carried out at 37 °C for 1 h. Alkylation was performed by adding 2 µL of iodoacetamide (400 mM in distilled water) for 1 h at room temperature in the dark. Urea concentration was lowered to 1 M with 50 mM AB, and protein digestion was performed overnight at 37 °C with 15 µL of freshly prepared trypsin Promega (0.2 µg/mL in AB). After bead removal, the sample was desalted with a C18 microspin column (Harvard Apparatus, Holliston, MA, USA), dried under speed-vacuum, and re-dissolved in H2O/CH3CN/FA 94.9/5/0.1 before liquid chromatography–electrospray ionisation–tandem mass chromatography (LC-ESI-MS/MS) analysis. LC-ESI-MS/MS analysis was performed on a Q-Exactive Hybrid Quadrupole-Orbitrap Mass Spectrometer (Thermo Fisher Scientific) equipped with an Easy nLC 1000 system (Thermo Fisher Scientific). Peptides were trapped on an Acclaim pepmap100, C18, 3 µm, 75 µm x 20 mm nano trap-column (Thermo Fisher Scientific) and separated on a 75 µm x 500 mm, C18, 2 µm Easy-Spray col- umn (Thermo Fisher Scientific). For the analytical separation was run for 90 min using a gradient of H2O/FA 99.9%/0.1% (solvent A) and CH3CN/FA 99.9%/0.1% (solvent B). The gradient was as follows: 0–5 min 95% A and 5% B, then to 65% A and 35% B over 35 min, 65% A and 35% B for 30 min, 95% A for 5 min, and 0% A and 100% B for 5 min.
Calcium measurements in T. b. berghei extracellular ookinete were fixed with 2.5% glutaraldehyde (Electron Microscopy Sciences) and 2.0% paraformaldehyde (Electron Microscopy Sciences) in 10 mM PBS pH 7.4 for 1 h at room temperature. Pelleted cells were embedded in 3% low melt agarose (Eurobio). Blocks of tissue were sectioned in small thin-wall PCR tubes and polyacrylamide gel electrophoresis and the main peak concentrated on 500 mL cultures in LB Rich Broth supplemented with 100 µg/mL ampicillin at 30 °C. The temperature was reduced to 16 °C before induction of expression with 1 mM IPTG. Incubation at 16 °C was continued overnight.

The cultures were separated by centrifugation (Beckman J25 with Fiberlite rotor at 80 °C for in excess of 1 h. The PKG’s were purified via the histidine tag on Hitrap TALON (cobalt) columns (GE Healthcare) connected to an AKTA-FPLC as per the manufacturer’s instructions. Fractions were analysed by sodium dodecyl sulphate–polyacrylamide gel electrophoresis and the main peak concentrated on 10 kDa molecular weight cut-off concentrators (Amicon). Purified proteins were stored in 50% glycerol at –80 °C in vials with aseptic aliquots. The final buffer consisting of the purified product was 50 mM Tris/HCl pH 7.5, 0.1 mM EDTA, 150 mM NaCl, 0.1% β-mercaptoethanol, 50% glycerol, 0.03% Brij-35, 1 mM benzamidine and 0.2 mM phenylmethylsulfonyl fluoride.

Microfluidic assay for recombinant cGMP-dependent protein kinase. The half-maximal inhibitory concentration (IC50) values were determined for test compounds using a microfluidic fluorescent shift assay described. Briefly, compounds were prepared over a 10-Well 1/2 log dilution series in DMSO in duplicate in 50 μL volumes using 384-well polypropylene U-bottomed plates (Thermo Scientific, UK). The plates contained positive/no inhibitor (DMSO only) and negative (no enzyme) controls in columns 1, 2 and 24. The reaction mix for each well consisted of 20 μL of enzyme/peptide mix (1.25 mM PKP, 1.5 mM FAM-labelled PKAtide (FAM-GRTGRNNI-NH2, Cambridge Bioscience, UK) in PKP assay buffer (25 mM Hepes (pH 7.4), 20 mM β-glycerophosphate, 2 mM DTT, 10 μM cGMP, 0.01% (v/v) bovine serum albumin (BSA), 0.01% (v/v) Triton X-100)) plus 5 μL of compound. Samples were pre-incubated at room temperature for 30 min and reactions were initiated by addition of 25-μL ATP mix (10 mM MgCl2 and ATP, at Km of enzyme under test (20 μM PKP and 90 μM PKP(76180)), in water). Positive controls were complete reaction mixtures with 10% DMSO and negative controls were reaction mixtures with 10% DMSO but lacking enzyme. Reactions were allowed to proceed for 30 min at room temperature, corresponding to conversion of approximately 10% of the substrate in the DMSO controls. Reactions were terminated by addition of 50 μL stop solution (25 mM EDTA in water). Samples were analysed by electrophoretic separation of substrate and product peaks and fluorescence detection using a Caliper Lab Chip EZ reader (Perkin Elmer, Waltham MA) with 0.2 s pipelime, downstream voltage 500 V, upstream voltage 1950 V and pressure 0.5 to 1.5 psi. Substrate and product peak heights were measured and the ratio of the product peak height divided by the sum of the product and substrate peaks were determined using EZ reader software (version 3.0.263.0) to obtain percentage conversion (P) values. P values were normalised to percentage activity relative to positive and negative controls were % activity = 100 × (P)100 – (Pneg ctrls)/(P pos ctrls – Pneg ctrls) and fitted to obtain IC50s using a 4-parameter logistic fit (LIDT, IBDS, Guildford UK). Liquid handling stages were conducted on a Biomek robotic liquid-handling Beckman Coulter).

Immunofluorescence labelling. Immunofluorescence assays were performed as described in ref. 35. Briefly, for HA staining, purified cells were fixed with 4% paraformaldehyde and 0.05% glutaraldehyde in PBS for 1 h, permeabilised with 0.1% Triton X-100/PBS for 10 min and blocked with 2% BSA/PBS for 2 h. Primary antibodies were diluted in blocking solution (rat anti-HA clone 3F10, 1:1000 from BioVendor, Ltd, Czech Republic). The plates contained positive/no inhibitor (DMSO only) and negative (no enzyme) controls in columns 1, 2 and 24. Excitation was measured using a SPECTRAmax microplate fluorescence reader (Molecular Devices, Sunnyvale, CA) with excitation and emission wavelengths of 490 nm and 520 nm, respectively. The plates were then washed with 2% BSA/PBS for 2 h and blocked with 2% BSA/PBS for 2 h. Primary antibodies were diluted in blocking solution (1:1000 from BioVendor, Ltd, Czech Republic). The plates were then washed with 2% BSA/PBS for 2 h and blocked with 2% BSA/PBS for 2 h. Primary antibodies were diluted in blocking solution (1:1000 from BioVendor, Ltd, Czech Republic). The plates were then washed with 2% BSA/PBS for 2 h and blocked with 2% BSA/PBS for 2 h. Primary antibodies were diluted in blocking solution (1:1000 from BioVendor, Ltd, Czech Republic). The plates were then washed with 2% BSA/PBS for 2 h and blocked with 2% BSA/PBS for 2 h. Primary antibodies were diluted in blocking solution (1:1000 from BioVendor, Ltd, Czech Republic). The plates were then washed with 2% BSA/PBS for 2 h and blocked with 2% BSA/PBS for 2 h. Primary antibodies were diluted in blocking solution (1:1000 from BioVendor, Ltd, Czech Republic). The plates were then washed with 2% BSA/PBS for 2 h and blocked with 2% BSA/PBS for 2 h. Primary antibodies were diluted in blocking solution (1:1000 from BioVendor, Ltd, Czech Republic). The plates were then washed with 2% BSA/PBS for 2 h and blocked with 2% BSA/PBS for 2 h. Primary antibodies were diluted in blocking solution (1:1000 from BioVendor, Ltd, Czech Republic).

**Data availability**

All relevant data are available from the authors on request. Mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD011096.
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