Plasmodium falciparum CRK4 directs continuous rounds of DNA replication during schizogony

Markus Ganter1, Jonathan M. Goldberg1, Jeffrey D. Dvorin1,21, Joao A. Paulo3, Jonas G. King4†, Abhai K. Tripathi4, Aditya S. Paul1, Jing Yang1, Isabelle Coppens4, Rays H. Y. Jiang3, Brendan Elsworth1, David A. Baker5, Rhel R. Dinglasan4†, Steven P. Gygi3 and Manoj T. Duraisingh1*

Plasmodium parasites, the causative agents of malaria, have evolved a unique cell division cycle in the clinically relevant asexual blood stage of infection1. DNA replication commences approximately halfway through the intracellular development following invasion and parasite growth. The schizont stage is associated with multiple rounds of DNA replication and nuclear division without cytokinesis, resulting in a multinucleated cell. Nuclei divide asynchronously through schizogony, with only the final round of DNA replication and segregation being synchronous and coordinated with daughter cell assembly2,3. However, the control mechanisms for this divergent mode of replication are unknown. Here, we show that the Plasmodium-specific kinase PfCRK4 is a key cell-cycle regulator that orchestrates multiple rounds of DNA replication throughout schizogony in Plasmodium falciparum. PfCRK4 depletion led to a complete block in nuclear division and profoundly inhibited DNA replication. Quantitative phosphoproteomic profiling identified a set of PfCRK4-regulated phosphoproteins with greatest functional similarity to CDK2 substrates, particularly proteins involved in the origin of replication firing. PfCRK4 was required for initial and subsequent rounds of DNA replication during schizogony and, in addition, was essential for development in the mosquito vector. Our results identified an essential S-phase promoting factor of the unconventional P. falciparum cell cycle. PfCRK4 is required for both a prolonged period of the intraerythrocytic stage of Plasmodium infection, as well as for transmission, revealing a broad window for PfCRK4-targeted chemotherapeutics.

Malaria parasites proliferate through schizogony in the blood stage of infection. A series of rapid rounds of DNA replication and nuclear division produces a syncytial cell with approximately 20 nuclei. In contrast to the synchronous nuclear division observed in other multinucleated cells, such as the early Drosophila embryo4, Plasmodium falciparum nuclei divide asynchronously during the blood stage, despite sharing the same cytoplasm2,3, suggesting that cell-cycle progression is not governed by diffusible cytoplasmic factors. Knockout screens have identified non-essential blood-stage Plasmodium kinases and suggested those that could be essential for the regulation of schizogony5,6. The molecular mechanisms regulating this diverged mode of replication are largely unknown, yet the recent development of conditional gene expression technologies now allows the unequivocal demonstration of gene essentiality in P. falciparum7–9.

To directly identify critical regulators of schizogony and simultaneously determine protein function, we adopted the destabilization domain (DD) conditional knockdown approach7,10,11. We generated endogenous DD fusions of 23 schizont-stage kinases in the P. falciparum D10 strain and screened for vulnerability to destabilization by DD (Fig. 1a,b and Supplementary Fig. 1a). Using this approach, we found that two kinases, the cGMP-dependent protein kinase (PfPKG) and the cdc2-related protein kinase 4 (PfCRK4), had profound proliferation defects in the absence of Shield-1 and showed dose dependency (Fig. 1c and Supplementary Fig. 1b,c). Chemical inhibition of PfPKG has previously established its essential role in parasite egress from erythrocytes at the end of schizogony12,13, now confirmed by conditional destabilization.

As previously observed14,15, we found little correlation between the level of protein knockdown and the ability to reveal the essentiality of other kinases previously thought to be essential16 using the DD approach (Fig. 1d and Supplementary Figs 1d and 9). This could be due to insufficient destabilization of these proteins or because they are required at different levels for asexual proliferation.

The biological function of PfCRK4 is unknown, and we confirmed its essentiality in a different parental line—P. falciparum P2G12 (ref. 16)—which also produces gametocytes (Supplementary Fig. 2). PfCRK4 is a member of an Apicomplexa-specific kinase subfamily related to cyclin-dependent kinases (CDK) (Supplementary Fig. 3 and Supplementary Data 1).17,18 In many organisms, CKDs in complex with cyclins regulate key steps of the cell cycle and other cellular functions19–21. Compared to human CDK2, the kinase domain of PfCRK4 possesses multiple sequence inserts of unknown function (Fig. 2a and Supplementary Fig. 4).

PfCRK4 is localized to the nucleus of late trophozoites and schizonts, with the signal greatly diminished in segmented schizonts that have undergone cytokinesis (Fig. 2b). Following conditional depletion of PfCRK4 (Fig. 1d), parasites arrested at the trophozoite-to-schizont transition (Fig. 2c). At ≥40 h post invasion (h.p.i.), parasites [+] Shield-1 (that is, wild-type PfCRK4 levels)
segmented into daughter cells containing nuclei and rhoptries (apical organelles required for merozoite invasion), which are characteristic of mature schizonts (Fig. 2d, top). In contrast, parasites cultured with Shield-1 (that is, PfCRK4 is depleted) showed no nuclear division or apical organelle biogenesis (Fig. 2d bottom and Supplementary Fig. 5a).

Analysis of nuclei stained with fluorescent DNA-specific dyes confirmed that the nuclei did not divide and revealed a substantially distorted nuclear morphology (Fig. 2e and Supplementary Fig. 5b). Hemispindle structures were evident in PfCRK4-depleted cells (Fig. 2f and Supplementary Fig. 5c,d); however, they were greatly enlarged relative to spindles in wild-type parasites and might account for the nuclear distortion. Concordantly, division of the centriolar plaque, the parasite’s microtubule organizing centre, was diminished (Fig. 2g). To ascertain whether PfCRK4 affects DNA replication, we quantified the parasite’s DNA content by flow cytometry. In wild-type parasites, DNA replication commences at 29–32 h.p.i. (that is, parasites with C-values >1 appear), but we found DNA replication to be profoundly inhibited in PfCRK4-depleted parasites (Fig. 2h). In contrast, the development of the mitochondria and apicoplast organelles was unaffected following depletion of PfCRK4 (Supplementary Fig. 5e,f). The PfCRK4-dependent block was completely reversible up to 38 h.p.i. by the addition of Shield-1. However, reversion at 48 h.p.i. led to very poor recovery (Fig. 2i), identifying a window within which PfCRK4 depletion is cytostatic before becoming cytotoxic.

To elucidate the processes regulated by PfCRK4, we assessed changes in the phosphoproteome of PfCRK4-depleted parasites at two time points (Fig. 3a): at 29 h.p.i., when PfCRK4 is already expressed at the onset of DNA replication (Fig. 2b,h) and also at 37 h.p.i., when in...
Figure 2 | Nuclear-localized PfCRK4 is essential for the trophozoite-to-schizont transition and DNA replication. a, Domain structure of PfCRK4. Blue, kinase domain; yellow, kinase domain insertions relative to human CDK2; red, predicted nuclear localization signal (NLS); asterisk, active site residues; 1 and 1,553, first and last amino acid (AA), respectively. b, Immunofluorescence detection of PfCRK4-HA-DD in blood-stage parasites (representative of three biological replicates). DIC, differential interference contrast. Scale bar, 3 μm. c, Light microscopy of May-Grünwald-Giemsa-stained PfCRK4-depleted parasites (D10 parent, representative of two biological replicates and results with P2G12-PfCRK4-HA-DD). Scale bar, 2 μm. d, Ultrastructure of PfCRK4-depleted parasites (D10 parent, representative of n ≥ 25 cells per condition). Arrowhead, spindle pole body; n, nucleus; Rh, rhoptry. Scale bars, 1 μm. e, Nuclear development in PfCRK4-depleted parasites (D10 parent, representative of three biological replicates, and results with P2G12-PfCRK4-HA-DD). Scale bar, 2 μm. f, Immunofluorescence detection of spindle structures in PfCRK4-depleted parasites (P2G12 parent, representative of two biological replicates). Tub, tubulin. Scale bar, 2 μm. g, Quantification of centriolar plaques by immunofluorescence in PfCRK4-depleted parasites (D10 parent; P value, chi-square test; representative of two biological replicates and results with P2G12-PfCRK4-HA-DD). Cen, centrin. Scale bar, 1 μm. h, DNA content of PfCRK4-depleted parasites from both parental lines, mean ± s.d. of technical triplicates (representative of three biological replicates); ring-stage DNA content defined as 1 (C-value). i, Proliferation of parasites when PfCRK4 was re-stabilized from 38 h.p.i. or 48 h.p.i. of the first cycle onwards; mean ± s.d. (representative of two biological replicates and results with P2G12-PfCRK4-HA-DD).

Figure 3 | PfCRK4 regulates S phase. a, Schematic illustrating the sampling time points for phosphoproteomic profiling. b, Differential phosphorylation in D10-PfCRK4-HA-DD parasites [−] Shield-1 relative to [+] Shield-1; 29 h.p.i. in duplicate, 37 h.p.i. in triplicate. P values, Student’s t-test. Grey-shaded regions indicate >2-fold changes in phosphorylation and P < 0.05. Numbers of peptides and respective proteins are indicated. c, GO term enrichment analysis of proteins with ≥2-fold-reduced phosphorylation (P < 0.05). P values, modified Fisher’s exact test. Box shows P. falciparum homologues of S. cerevisiae factors required for origin of replication activation in vitro with reduced phosphorylation at 37 h.p.i. in PfCRK4-depleted parasites. Pol, DNA polymerase; MCM, mini-chromosome maintenance complex; TOP2, topoisomerase 2; RPA, replication protein A; ORC, origin recognition complex; eIF3, eukaryotic initiation factor 3. d, Percentage of shared GO terms of proteins with ≥2-fold-reduced phosphorylation (P < 0.05) upon PfCRK4-depletion and of substrate sets of human and S. cerevisiae (Sc) kinases.
An. gambiae gametocytogenesis diminished oocyst numbers on ≥ of (Kruskal midguts. Bars, mean oocyst numbers; NS, not significant of cultures from a (representative of Pf strains). b) Effect of CRK4-depleted parasites (Fig. 3c box, Supplementary Fig. 6f and Supplementary Data 3 and 4). GO terms of proteins with a ≥2-fold increase in phosphorylation (P ≤ 0.05) were enriched for biosynthetic and intracellular transport processes at 37 h.p.i., perhaps indicating secondary effects, but no statistically significant enrichment was observed at 29 h.p.i. (Supplementary Fig. 6g). We next compared the GO terms of the PfCRK4-regulated set with GO terms of other available kinase substrate sets—one S. cerevisiae kinase and 19 human kinases (www.phosphosite.org)—each with >30 confirmed substrates (Fig. 3d). The substrate set of human CDK2, a major S-phase promoting factor, showed the highest percentage of shared annotations, suggesting that PfCRK4 acts in a related fashion to promote the S phase in P. falciparum.

PfCRK4 protein levels increased through early and mid schizogony (Fig. 2b) and its transcripts peak at the latest of all putative Plasmodium CDK-like kinases. We therefore hypothesized that PfCRK4 is crucial for all rounds of DNA replication seen in schizogony. When we depleted PfCRK4 at points later in schizogony, we detected no further increase in DNA content (Fig. 4a), premature termination of nuclear division (Fig. 4b), and the parasites failed to proliferate (Fig. 4c), thus demonstrating that PfCRK4 function is critical throughout schizogony.

To determine a role for PfCRK4 in other life-cycle stages, we used the P2G12-PfCRK4-HA-DD line that produces high levels of gametocytes to analyse PfCRK4 function during parasite transmission to the Anopheles gambiae mosquito vector. Although we detected nuclear expression of PfCRK4 in gametocytes (Fig. 4d and Supplementary Fig. 7a), depletion during the latter portion of gametocyte development (that is, from day 6 to day 16 post-induction) had no effect on gametocytemia nor on the male-to-female gametocyte ratio (Supplementary Table 1). PfCRK4 depletion also did not impair exflagellation (Supplementary Fig. 7b–d). We observed a reduced PfCRK4 signal in ookinetes (Supplementary Fig. 7e), and the numbers of oocysts on An. gambiae female midguts was greatly diminished following PfCRK4 depletion (Fig. 4e and Supplementary Fig. 8). Oocysts derived from gametocyte cultures in the presence of PfCRK4 showed multiple nuclei, well-expanded organelles and a well-developed capsule (Fig. 4f, top). In contrast, infections with PfCRK4-depleted gametocytes resulted in necrotic parasites reminiscent of dead oocysts, the motile zygote of Plasmodium parasites (Fig. 4f bottom and Supplementary Fig. 8). This indicates that PfCRK4 is required at some point during ookinete formation and early oocyst development, and might play a role during DNA replication in zygotes.

Our data provide evidence that PfCRK4 is an essential protein kinase promoting S phase through the initiation of multiple rounds of DNA replication during schizogony. Phosphorylation is probably important for the regulation of PfCRK4 (Supplementary Figs 3b and 4a), but a potential cyclin dependence is in question, as the cyclin-binding domain is poorly conserved (Supplementary Fig. 4a)27. Interestingly, homologues of Group I cyclins are not found in the Plasmodium genome, including cyclin E, which is critical for the G1/S transition in other organisms28. Interventions targeting PfCRK4 function would be efficacious throughout the extended period of schizogony in the clinically relevant blood stage, as well as in the transmission stages, which taken together are attractive features for future drug development.

Methods
Reagents and oligonucleotide primers. We purchased chemicals from Sigma-Aldrich (unless otherwise noted), primers from Integrated DNA Technologies (sequences are available upon request) and restriction enzymes from New England Biolabs.

Figure 4 | PfCRK4 is essential throughout schizogony and critical for transmission. a, DNA content analysis of D10-PfCRK4-HA-DD parasites when PfCRK4 was present or depleted from 32 h.p.i. or 36 h.p.i. onwards. 1 (C-value), ring-stage DNA content; *asterisks, time of Shield-1 removal (representative of two biological replicates and results with P2G12-PfCRK4-HA-DD). Note: second-cycle ring-stage parasites reappear at 48 h.p.i. when PfCRK4 is present. b, DAPI-stained nuclei of parasites from b (representative of n ≥70 cells per condition). Scale bar, 2 μm. c, Proliferation of cultures from a (mean ± s.d. of triplicates). d, Immunofluorescence detects PfCRK4-HA-DD in gametocytes (P2G12 parent, representative of two biological replicates). Scale bar, 4 μm. e, Depletion of PfCRK4 during gametocytogenesis diminished oocyst numbers on An. gambiae female midguts. Bars, mean oocyst numbers; NS, not significant; **P ≤ 0.01 (Kruskal-Wallis test). f, Ultrastructure of mosquito-stage parasites from infections with PfCRK4-depleted gametocytes (P2G12 parent, representative of ≥10 cells from independent infection experiments). ER, endoplasmic reticulum; n, nucleus; m, mitochondria; c, capsule. Scale bars, 1 μm.
Construction of plasmids. The terminal 1–2 kb of PKB (PF3D7_1246900), TLK4 (PF3D7_0623000) and PKG (PF3D7_1436600) were subcloned into the 3x replacement vector pJJDD14 (ref. 7), generating HA-DD single-crossover tagging plasmids. The terminal ~1 kb of PF3D7_0420100 was amplified using an extended reverse primer, introducing two additional HA-tags. Cloning into pJJDD1 resulted in a 3xAHA-DD single-crossover tagging plasmid. All other targeting fragments were subcloned into this 3xAHA-DD plasmid (sequences available upon request).

Parasite culture, transfection and synchronization. P. falciparum D10 was obtained from the Walter and Eliza Hall Institute (Melbourne, Australia). P. falciparum PGZ12 (clone of 3D7) was obtained from Harvard T.H. Chan School of Public Health (Boston, USA). Parasites were cultured as previously described in RPMI 1640 medium supplemented with 0.5% Albumax-II (Invitrogen), 50 mg l⁻¹ hypoxanthine, 0.21% sodium bicarbonate and 25 mM HEPES (EMD Biosciences) 31. Human O+ erythrocytes (Research Blood Components) were diluted to 2–4% haematocrit. Transgenic parasites were generated by electroporation of synchronization ring-stage parasites as previously described 32. Single homologous recombination events were selected in the presence of 500 nM Shield-1 by cycling WR99210 (Jacobus Pharmaceutical Company) on and off, and cloned by limiting dilution. Parasites were synchronized by a combination of heparin and sorbitol treatments as previously described 33. Unless otherwise noted, DD-tagged parasites were cultured in 250 nM Shield-1. When desired, Shield-1 was removed by triple washes with excess RPMI.

Southern blot analysis. Genomic DNA was collected with a QiAamp Blood Mini Kit (Qiagen) and digested with the enzymes indicated in Supplementary Fig. 1a, resolved on 0.8% agarose gels, transferred to GeneScreen Plus (Perkin Elmer) and hybridized with specific radio-labelled probes.

Malaria—Grimwald–Giemsa staining and imaging of blood-stage parasites. Air-dried thin-smear blood films were fixed and stained according to manufacturers’ instructions and imaged on a Zeiss AxioCam microscope equipped with a ×100 oil-immersion objective. Raw images were analysed using ImageJ (ref. 34). Dose response experiments. Dose response curves were generated as previously described using SYBR Green 1 (ref. 35). In brief, triplicate twofold Shield-1 dilution series were set up in 100 µl parasite cultures (0.075% parasitaemia) in 2% BSA in 3 ml RPMI 1640. Following 20 min incubation at 37 °C, parasites were washed, spotted on a glass slide, air-dried, methanol-fixed and processed further as described above.

Immunofluorescence assays. Immunofluorescence assays of blood-stage parasites was carried out as previously described 37,38. In brief, parasitized erythrocytes were air-dried, methanol-fixed 38, and analysed using rat anti-HA (1:100, Roche, clone 3F10), mouse anti-centrin (1:500, Millipore, clone 20H5), mouse anti-a-tubulin (1:2,000, Sigma-Alrich, clone B-5–1–2), rabbit anti-PfKAP45 (1:2,000 dilution, from J.G. Rayner) or rabbit anti-PfER175 (1:500, Malaria Research and Reference Reagent Resource Center, R3347) antibodies. Alternatively, parasites were fixed in solution 39 for 20 min at room temperature, immobilized on poly-L-lysine coated coverslips (Corning), and analysed using mouse anti-a-tubulin (1:2,000, Sigma-Alrich, clone B-5–1–2) or rabbit anti-PfJCCP 1–2 (1:500, from S.T. Prigge) antibodies. Primary antibodies were detected using Alexa Fluor–conjugated secondary antibodies (1:2,000, Thermo Fisher Scientific). Cells were mounted with DAPI (4,6-diamidino-2-phenylindole) Fluoromount-G (SouthernBiotech) or cells were stained with Hoechst 33342 (Thermo Fisher Scientific) and mounted with Fluoromount-G (SouthernBiotech), imaged on a Nikon Eclipse TE300 microscope equipped with a Hamamatsu C10600 Orca R2 digital camera, and analysed using ImageJ software.

For immunofluorescence analysis of exflagellation, mature gametocytes were resuspended in RPMI 1640 medium supplemented with 20% human serum (AB+), 50 mg l⁻¹ hypoxanthine, 0.21% sodium bicarbonate, 25 mM HEPES (EMD Biosciences) and 50 µM xanthurenic acid at room temperature and pH 8 to induce exflagellation 40. Following 20 min incubation at room temperature, cells were washed, spotted on a glass slide, air-dried, methanol-fixed and processed further as described above.

For immunofluorescence analysis of ookinetes, female An. gambiae mosquitoes were fed with late-stage PfCRK4-HA-DD gametocyte cultures, which were maintained (+) and −Shield-1 from day 6 post-induction. Approximately 24 h post-feeding, the blood bolus from the mosquito midgut lumen was isolated. Bolus material was spotted on glass slides and air-dried before immunofluorescence analysis.

Immunofluorescence analysis of P. falciparum late midgut oocysts was done as previously described 37. In brief, dissected midguts were fixed in 4% paraformaldehyde for 1 h at room temperature, washed, permeibilized and blocked using 0.2% Triton X-100 in 1% bovine serum albumin in PBS. Midgut oocysts were analysed using mouse anti-circumsporozoite protein (CS) antibody (15 µg ml⁻¹, gift of P. Sinis), an Alexa Fluor 594 secondary antibody (1:1,000) and DAPI nuclear stain, mounted on a glass slide in 10% glycerol in PBS and imaged on a Nikon Eclipse 90i microscope. Raw images were analysed using Velocity software (Perkin–Elmer).

JC-1 staining of P. falciparum mitochondria. Parasite mitochondria were stained with the cationic mitochondrial membrane potential sensor JC-1 (Life Technologies) as previously described 41. In brief, 2 µM JC-1 in 37 °C warm RPMI was filtered using an Acrodisc Syringe Filter (0.2 µm HT Tuffryn Membrane, Pall) to remove preformed JC-1 aggregates. Parasites were incubated with JC-1-containing medium for 20 min at 37 °C, washed, and imaged immediately on a Nikon Eclipse TE300 microscope equipped with a Hamamatsu C10600 Orca R2 digital camera and analysed using ImageJ software.

Electron microscopy. Synchronized blood-stage parasites were isolated by magnetic affinity purification using a MACS LS column (Miltenyi Biotec), adjusted to ~50% parasitaemia with infected erythrocytes and resuspended in 200 µl 3% BSA in PBS. Cells were fixed for 1 h at room temperature with an equal volume of 2.5% paraformaldehyde, 5.0% glutaraldehyde and 0.06% picric acid in 0.2 M cacodylate buffer.
buffer. Cells were washed in ccaday buffer, post-fixed for 1 h with 1% osmium tetroxide (OsO4), further dehydrated in 50% acetone and 50% potassium ferrocyanide (KFeCN6) and washed in H2O. Following 1 h incubation in 1% aqueous uranyl acetate solution, samples were washed in H2O and subsequently dehydrated in grades of alcohol. The samples were placed in propyleneoxide for 1 h and infiltrated in a 1:1 mixture of propyleneoxide and TAAB Epon (Marivac) over night. Samples were embedded in TAAB Epon and polymerized at 60°C for 48 h. Ultrathin sections from a Reichert Ultracut E microtome were mounted on copper grids and stained with lead citrate. Samples were imaged on a Tecnai G2 Spirit BioTWIN with an AMT 2k charge-coupled device camera under 80 kV.

For P. falciparum oocyst thin-section transmission electron microscopy, infected midguts were fixed at day 7 post-infection in 2.5% glutaraldehyde (Electron Microscopy Sciences) and processed as described in ref. 44. Stained sections were examined with a Philips CM120 electron microscope under 80 kV.

Phosphoproteomic profiling

Cell culture and collection. Synchronous D10-PcCRK4-HA-DD parasites were cultured for 29 or 37 h.p.i. [+] or [-]. At 29 h.p.i., duplicate samples were collected, and at 37 h.p.i., triplicate samples were collected for both [+ and -] Shield-1. For each of the ten samples, ~12.5 x 10^7 parasites were isolated, and uninfected erythrocytes were removed using saponin buffer (0.05% saponin in PBS plus 1x PhosSTOP (Roche), 1x Complete protease inhibitor cocktail (Roche) and 1 mM phenylmethylsulfonyl fluoride (PMSF)). Subsequently, cells were washed in saponin buffer to further remove erythrocytes and erythrocyte debris, followed by washes with PBS plus 1x PhosSTOP, 1x Complete protease inhibitor cocktail and 1 mM PMSF.

Cell lysis and protein digestion. Lysates were prepared as previously described25,45. In brief, cells were lysed in 8 M urea, 100 mM sodium chloride, 25 mM TRIS pH 8 plus 1x PhosSTOP, 1x Complete protease inhibitor cocktail and 1 mM PMSF in PBS. The protein concentration was estimated in the Bio-Rad Protein Assay (Bio-Rad). Dialysed buffer was reduced with 5 mM tris(2-chloroethyl) phosphate (TCEP) for 30 min at 37 °C. Cysteines were alkylated with 15 mM iodoacetamide for 30 min at room temperature in the dark, followed by incubation with 5 mM dithiothreitol (DTT) for 15 min at room temperature in the dark to quench excess iodoacetamide. Chloroform-methanol precipitated proteins were resuspended in 8 M urea, 50 mM HEPES pH 8.5 and subsequently diluted to 1 M urea 50 mM HEPES pH 8.5 for digestion with 100 µg/ml trypsin (1:100 protease-to-protein ratio, 3 h at 37 °C) before the addition of trypsin (1:100 protease-to-protein ratio) and continued digest overnight at 37 °C. The reaction was quenched with 1% formic acid, subjected to C18 solid-phase extraction (Seph-Pak, Waters), followed by vacuum-centrifugation.

Isobaric labelling with tandem mass tags (TMTs). Peptides (200 µg) from each sample were dissolved in 100 mM HEPES pH 8.5. Labelling with TMT reagents (Thermo Fisher Scientific) was carried out according to the manufacturer’s instructions and as previously described46. The TMT-labelled samples were combined in a 1:1 ratio across all samples, vacuum centrifuged to near dryness, and subjected to C18 solid-phase extraction (Seph-Pak, Waters), followed by vacuum-centrifugation.

Phosphopeptide enrichment. Peptides were resuspended in 100 mM HEPES pH 8.5 and phosphopeptides were enriched using Titanosil TiO2, 5 µm particles (GL Biosciences) as previously described47,48. The flow-through was kept for proteome analysis. Enriched TMT-labelled phosphopeptides and peptides were dried by vacuum-centrifugation.

Offline basic pH reversed-phase (BPRP) fractionation. For protein level analysis, we fractionated the pooled TMT-labelled peptide sample via BPRP high-performance liquid chromatography as previously described49,50. Fractions were desalted, dried and reconstituted in 5% acetonitrile, 5% formic acid for mass spectrometry processing.

For phosphopeptide analysis, we used the Pierce Off-line BPRP fractionation kit (Thermo Scientific). Fractions collected included organic buffer bumps with 5, 7.5, 10, 12.5, 15, 17.5, 20, 30 and 70% acetonitrile, in addition to the wash and flow through fractions. Fractions were vacuum-centrifuged to near dryness, desalted via StageTip, dried and reconstituted in 5% acetonitrile/5% formic acid for LC-MS/MS processing.

Liquid chromatography and tandem mass spectrometry. Data were collected using an Orbitrap Fusion mass spectrometer (Thermo Fisher Scientific) coupled to a PicoTec EASY-ESiy-NLC II liquid chromatography (LC) pump (Thermo Fisher Scientific). Peptides were fractionated on a 75 µm inner diameter microcapillary column packed with ~0.5 cm Magic C4 resin (5 µm, 100 Å, Michrom Biosources) followed by ~35 cm GP-18 resin (1.8 µm, 200 Å, Sepax). For each analysis, we loaded ~1 µg onto the column per analysis. For total proteome analysis, peptides were separated using a 180 min gradient of 6–23% acetonitrile in 0.125% formic acid (flow rate of ~600 nl min^-1). Each analysis used the multi-notch MS3-based TMT method51. The scan sequence began with an MS1 spectrum (Orbitrap resolution; resolution of 120,000, mass range of 400–2000 m/z); the mass and gain control (AGC) target of 2.0 x 10^6, maximum injection time of 100 ms). Precursors for MS2/MS3 analysis were selected using a TopSpeed 2 s method. MS2 analysis consisted of collision-induced dissociation (CID) mass range of 450–1400 m/z; isolation width of 1.10,10,100. NCE of 35, maximum injection time of 150 ms). Following acquisition of each MS2 spectrum, we collected an MS3 spectrum using a recently described method in which multiple MS2 fragment ions were captured in the MS3 precursor population using isolation waveforms with multiple frequency notches52. MS3 precursors were fragmented by high-energy collision-induced dissociation (HCD) and analysed (NCE 55: AGC: of 5 x 10^6, maximum injection time of 150 ms, resolution of 60,000 at 200 Th).

For phosphoproteome analysis, peptides were separated using a 120 min gradient of 3–23% acetonitrile in 0.125% formic acid (flow rate of ~575 nl min^-1). Each analysis used the multi-notch MS3-based TMT method51. The scan sequence was identical to the proteome scan, except the maximum injection time was 200 ms for MS2 analysis and 250 ms for MS3 analysis.

Initial mass spectrometry data analysis. Mass spectra were processed using a SEQUEST-based in-house software pipeline52. Spectra were converted to mzXML using a modified version of ReAdW.exe. Database searching included all entries from a combined Uniprot human and Plasmodium database (February 2015). This database was concatenated with one composed of all protein sequences in the reversed order. Total protein level analysis searches were performed using a 50 ppm precursor ion tolerance, and product ion tolerance was set to 0.9 Da. These wide mass tolerance windows were chosen to maximize sensitivity in conjunction with SEQUEST and linear discriminant analysis53,54. TMT tags on lysine residues and peptide N termini (+229.16 Da) and carbamidomethylation of cysteine residues (+57.02 Da) were set as static modifications, while oxidation of methionine (+15.995 Da) was set as a variable modification for total proteome and phosphoproteome data sets. For phosphoprotein analysis, +79.966 Da was set as a variable modification on serine, threonine and tyrosine residues.

Pepitde-spectral matches (PSMs) were adjusted to a 1% false discovery rate (FDR)26,55. PSM filtering used a linear discriminant analysis as previously described48, while considering the following parameters: XCorr, ΔCn. missed cleavages, peptide length, charge state and precursor mass accuracy. For the phosphorylation data set, site localization was evaluated via AScore53. For TMT-based reporter ion quantitation, we extracted the summed signal-to-noise (S/N) ratio for each TMT channel and found the closest matching centroid to the expected mass of the TMT reporter ion.

The search space for each reporter ion was limited to a range of 0.003 Th to prevent overlap between the isobaric reporter ions. For protein-level comparisons, PSMS were identified, quantified, collapsed to a 1% peptide FDR, and further collapsed to a final protein-level FDR of 1%. Protein assembly was guided by principles of parsimony to produce the smallest set of proteins necessary to account for observed peptides.

Proteins were quantified by summing reporter ion counts across all matching PSMS using in-house software, as previously described25. Poor-quality PSMS, MS3 spectra with >8 TMT reporter ion channels missing, MS3 spectra with TMT reporter summed S/N ratio <100, or no MS3 spectra were excluded from quantitation. Protein quantitation values were exported for further analysis. Excel or SAS JMP. Each reporter ion channel was summed across all quantified proteins and normalized assuming equal protein loading of all ten samples.

Evolutionary relationship of PfCRK4. Protein kinase amino acid sequences from the cycload-dependent kinase (CDK) families of apicomplexans and model organism and non-CDK out-group kinases were chosen for comparison with PfCRK4 (Supplementary Data 1). Kinase domain sequences were trimmed, aligned using MUSCLE56 and analysed using RAXML57.

Domain drawings of PfCRK4 and its orthologues in Plasmodium spp. Domain boundaries were identified by searching sequences against Pfam58 using the HMMER package59. Domains were illustrated using Protdraw (http://sourceforge.net/projects/protdraw/).

Homology modelling of PfCRK4. The protein kinase domain of PfCRK4 was modelled using the co-crystal structure of the human CDK2-cyclin A-substrate peptide complex (1IQM.pdb)35. We used this structure as a template to model PfCRK4 in a potentially active form. The kinase domains of the target and query sequences were aligned using MUSCLE56 followed by manual review and adjustment. Homology models were made using MODELLER 9.15 (ref. 60). Structural alignments were reviewed and adjusted using Chimera41.

Identification of conditionally related clusters in proteomic and phosphoproteomic data. The total sum S/N values of peptides collapsed into Supplementary Data 3. For phosphopeptides values see Supplementary Data 4. Contaminants and false positives were removed and values for each channel were normalized by the column sum. Values were placed on a scale of 0–1 by dividing by the maximum value for the protein or phosphopeptide. Scaling was done for all channels at once and separately for experiments at 29 h.p.i. and 37 h.p. Phosphopeptides for a total sum S/N <100 were not included in clustering analysis and the total sum S/N values were adjusted to reflect protein levels from the
proteome analysis. K-means clustering\(^6\) was done using R (http://cran.r-project.org/) with the Hartigan–Wong method using 1,000 starting configurations and 10,000 iterations. Clustering results, log_{10}-fold changes, and \(-log_{10} P\) values (Student’s t-test) are included in Supplementary Data 3 and 4.

### Gene orthology relationships

Relationships between *P. falciparum* proteins and *S. cerevisiae* orthology are shown in Table 2 (Supplementary Data 3). The experiment was investigated using a downloaded version of the OrthoMCL database, version 5 (ref. 63). A comprehensive orthology analysis is provided in Supplementary Data 6.

### Functional annotation and enrichment analysis

The functional enrichment of sets of proteins with reduced abundance in the absence of Shield-1 was manually inspected. The motifs discernable in this set may be retrieved from tab-delimited tables of triskaidekapeptides by the regular expressions \[A-Z\]{6}\[ST\].

### Comparative phosphoproteomics

Phosphopeptides with reduced abundance in the absence of Shield-1 were manually inspected. The substrate set from the reference kinases was divided by the number of significant functional terms in the substrate set from the reference kinase.

### Identification and enrichment analysis of phosphorylation motifs

Alignments of phosphopeptides with reduced abundance in the absence of Shield-1 were manually inspected. The two motifs discernable in this set may be retrieved from tab-delimited tables of triskaidekapeptides by the regular expressions \[A-Z\]{6}\[ST\].

### Data availability

The data supporting the results of this study are available within the paper and its Supplementary Information and Supplementary Data. Sequences of oligonucleotides and plasmids generated for this study are available from the corresponding author upon request.

Received 29 May 2016; accepted 13 January 2017; published 17 February 2017; corrected 6 March 2017

### References

1. Francia, M. E. & Striepen, B. Cell division in apicomplexan parasites. *Nat. Rev. Microbiol.* 12, 125–136 (2014).

2. Read, M., Sherwin, T., Holloway, S. P., Gull, K. & Hyde, J. E. Microtubular organization visualized by immunofluorescence microscopy during erythrocytic schizogony in *Plasmodium falciparum* and investigation of post-translational modifications of parasite tubulin. *Parasitology* 106, 223–232 (1993).

3. Arnott, D. E., Ronander, E. & Bengtsson, D. C. The progression of the intra-erythrocytic cycle of *Plasmodium falciparum* and the role of the centroplar plaques in asynchronous mitotic division during schizogony. *Int. J. Parasitol.* 41, 71–80 (2011).

4. Farrell, J. A. & O’Farrell, P. H. From egg to gastrula: how the cell cycle is involved in pre-S-phase development. *Proc. Natl Acad. Sci. USA* 107, 1054–1059 (2009).

5. Coudreuse, D. & Nurse, P. Driving the cell cycle with a minimal CDK control module. *Genes Dev.* 15, 1791–1806 (2001).

6. Lowe, E. D. et al. Specificity determinants of recruitment peptides bound to phospho-CRCK2/cyclin A. *Biochemistry* 41, 15625–15632 (2002).

7. Ubersax, J. A. et al. The CRK4-regulated set of phosphoproteins. *J. Cell Sci.* 14, 315–325 (2002).

8. Koopman, B. et al. A novel set of transfection vectors and a new immunofluorescence assay for use in malaria drug screening. *PLoS Pathog.* 11, e1005273 (2015).

9. Assumani, T. et al. Assessment and continued validation of the malaria SYBR green I-based fluorescence assay for use in malaria drug screening. *Antimicrob. Agents Chemother.* 51, 1926–1933 (2007).

10. Neuhauer, C. *Calculs For Biology and Medicine: Pearson New International Edition* (Pearson, 2013).

11. Tonkin, C. J. et al. Localization of organellar proteins in *Plasmodium falciparum* using a novel set of transferrin vectors and a new immunofluorescence fixation method. *Mol. Biochem. Parasitol.* 137, 13–21 (2004).

12. Dinglasan, R. R. et al. *Plasmodium falciparum* ookinete requires mosquito midgut chondroitin sulfate proteoglycans for cell invasion. *Proc. Natl Acad. Sci. USA* 104, 15882–15887 (2007).

© 2017 Macmillan Publishers Limited. part of Springer Nature. All rights reserved.
39. Flueck, C. et al. A major role for the Plasmodium falciparum ApiAP2 protein PSIP2 in chromosome end biology. PLoS Pathog. 6, e1000784 (2010).
40. Gallagher, J. R., Matthews, K. A. & Prigge, S. T. Plasmodium falciparum apicoplast transit peptides are unstructured in vitro and during apicoplast import. Traffic 12, 1124–1138 (2011).
41. Billek, O. et al. Identification of xanthurenic acid as the putative inducer of malaria development in the mosquito. Nature 392, 289–292 (1998).
42. Thathy, V. et al. Levels of circumsporozoite protein in the Plasmodium oocyst determine sporozoite morphology. EMBO J. 21, 1586–1596 (2002).
43. Pasini, E. M., van den Ijssel, D., Vial, H. J. & Kocken, C. H. M. A novel live-dead staining methodology to study malaria parasite viability. Malar. J. 12, 190 (2013).
44. Coppen, J. & Joiner, K. A. Host but not parasite cholesterol controls toxoplasma cell entry by modulating organelle discharge. Mol. Biol. Cell 14, 3804–3820 (2003).
45. Villén, J. & Gygi, S. P. The SCX/IMAC enrichment approach for global phosphorylation analysis by mass spectrometry. Nat. Protoc. 3, 1630–1638 (2008).
46. Paulo, J. A. et al. Effects of MEK inhibitors GSK1120212 and PD0325901 in vivo using 10-plex quantitative proteomics and phosphoproteomics. Proteomics 15, 462–473 (2014).
47. Wessel, D. & Flügge, U. I. A method for the quantitative recovery of protein in dilute solution in the presence of detergents and lipids. Anal. Biochem. 138, 141–143 (1984).
48. Paulo, J. A. & Gygi, S. P. A comprehensive proteomic and phosphoproteomic analysis of yeast deletion mutants of 14-3-3 orthologs and associated effects of rapamycin. Proteomics 15, 474–486 (2015).
49. Kettenbach, A. N. & Gerber, S. A. Rapid and reproducible single-stage phosphopeptide enrichment of complex peptide mixtures: application to general and phosphotyrosine-specific phosphoproteomics experiments. Anal. Chem. 83, 7635–7644 (2011).
50. Paulo, J. A., Gaun, A. & Gygi, S. P. Global analysis of protein expression and phosphotyrosine-specific phosphoproteomic experiments. Nat. Biotechnol. 24, 1285–1292 (2006).
51. Elias, J. E. & Gygi, S. P. Target-decoy search strategy for increased confidence in large-scale protein identifications by mass spectrometry. Nat. Methods 4, 207–214 (2007).
52. Elias, J. E. & Gygi, S. P. Target-decoy search strategy for mass spectrometry-based proteomics. Methods Mol. Biol. 604, 55–71 (2010).
53. Edgar, R. C. MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res. 32, 1792–1797 (2004).
54. Stamatakis, A. RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. 30, 1312–1313 (2014).
55. Finn, R. D. et al. Pfam: the protein families database. Nucleic Acids Res. 42, D222–D230 (2014).
56. Huttlin, E. L. Increasing the multiplexing capacity of TMTs using reporter ion isotopologues with isobaric masses. Anal. Chem. 84, 7469–7478 (2012).
57. Beausoleil, S. A., Villén, J., Gerber, S. A., Rush, J. & Gygi, S. P. A probability-based approach for high-throughput protein phosphorylation analysis and site localization. Nat. Biotechnol. 24, 1285–1292 (2006).
58. Finn, R. D. et al. HMMER web server: 2015 update. Nucleic Acids Res. 43, W30–W38 (2015).
59. Webb, B. & Sali, A. Comparative protein structure modeling using MODELLER. Curr. Protoc. Bioinformatics 47, 5–32 (2014).
60. Pettersen, E. F. et al. UCSF Chimera—a visualization system for exploratory research and analysis. J. Comput. Chem. 25, 1650–1652 (2004).
61. Beausoleil, S. A., Villén, J., Gerber, S. A., Rush, J. & Gygi, S. P. Target-decoy search strategy for increased confidence in large-scale protein identifications by mass spectrometry. Nat. Methods 4, 207–214 (2007).
62. Crooks, G. E., Hon, G., Chandonia, J.-M. & Brenner, S. E. Weblogo: a sequence logo generator. Genome Res. 14, 1188–1190 (2004).
Erratum: *Plasmodium falciparum* CRK4 directs continuous rounds of DNA replication during schizogony

Markus Ganter, Jonathan M. Goldberg, Jeffrey D. Dvorin, Joao A. Paulo, Jonas G. King, Abhai K. Tripathi, Aditya S. Paul, Jing Yang, Isabelle Coppens, Rays H. Y. Jiang, Brendan Elsworth, David A. Baker, Rhoel R. Dinglasan, Steven P. Gygi and Manoj T. Duraisingh

Nature Microbiology 2, 17017 (2017); published online 17 February 2017; corrected 6 March 2017.

In the version of this Letter originally published, the in-text citations to Supplementary Table 1 and Supplementary Data 1–7 were ambiguous. This has now been corrected in all versions of the Letter so that there is a clear distinction between the different files, with citations to Supplementary Table 1 and Supplementary Data 1–7 now appearing in the text. In addition, the definition of DIC in Fig. 2 was placed incorrectly. This has now been moved to Fig. 2b, in keeping with the figure panels.