Mapping and functional analysis of heterochromatin protein 1 phosphorylation in the malaria parasite *Plasmodium falciparum*

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Previous studies in model eukaryotes have demonstrated that phosphorylation of heterochromatin protein 1 (HP1) is important for dynamically regulating its various functions. However, in the malaria parasite *Plasmodium falciparum* both the function of HP1 phosphorylation and the identity of the protein kinases targeting HP1 are still elusive. In order to functionally analyze phosphorylation of *P. falciparum* HP1 (PFHP1), we first mapped PFHP1 phosphorylation sites by liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis of native PFHP1, which identified motifs from which potential kinases could be predicted; in particular, several phosphorylated residues were embedded in motifs rich in acidic residues, reminiscent of targets for *P. falciparum* casein kinase 2 (PfCK2).

Secondly, we tested recombinant PfCK2 and a number of additional protein kinases for their ability to phosphorylate PFHP1 in *in vitro* kinase assays. These experiments validated our prediction that PFHP1 acts as a substrate for PfCK2. Furthermore, LC-MS/MS analysis showed that PfCK2 phosphorylates three clustered serine residues in an acidic motif within the central hinge region of PFHP1. To study the role of PFHP1 phosphorylation in live parasites we used CRISPR/Cas9-mediated genome editing to generate a number of conditional PFHP1 phosphomutants based on the DiCre/LoxP system. Our studies revealed that neither PfCK2-dependent phosphorylation of PFHP1, nor phosphorylation of the hinge domain in general, affect PFHP1’s ability to localize to heterochromatin, and that PFHP1 phosphorylation in this region is dispensable for the proliferation of *P. falciparum* blood stage parasites.

Euchromatin and heterochromatin are the two main structures of chromatin in eukaryotes. While euchromatin is associated with active gene transcription, heterochromatin is associated with heritable gene silencing. Heterochromatin is characterized by the enrichment of heterochromatin protein 1 (HP1) bound to trimethylated histone 3 lysine 9 (H3K9me3)1–3. HP1 recruits chromatin modifiers such as H3K9me-specific histone methyltransferases, which in turn methylate H3K9 in neighbouring nucleosomes, thus facilitating the binding of further HP1 proteins and consequently the regional spreading of heterochromatin in a sequence-independent manner3,4. In addition to promoting gene silencing and heterochromatin maintenance HP1 also plays roles in centromere function in fission yeast and humans and in DNA replication and repair3,6.

HP1 is widely conserved among eukaryotes and consists of three functional domains, namely the N-terminal chromo domain (CD) that binds H3K9me31,7,8, the C-terminal chromoshadow domain (CSD) that mediates HP1 homodimerisation and specific interactions with other regulatory proteins9–11, and a variable hinge region located between the CD and CSD domains that has been shown to interact with DNA and/or RNA12–14. Some eukaryotes have several HP1 paralogs; for instance, *Schizosaccharomyces pombe* encodes two HP1 variants (Swi6 and Chp2) and mammals possess three HP1 variants (HP1α, HP1β and HP1γ)3,5.

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The parasitic protist *Plasmodium falciparum*, the causative agent of the most severe form of malaria in humans, possesses a single H1 ortholog (PfHP1). PfHP1 binds to and co-localizes with H3K9me3 to heterochromatic domains in the subtelomeric regions of all 14 chromosomes and to internal heterochromatic islands on some chromosomes15–19. There is no evidence for the presence of either PfHP1 or H3K9me3 in peri-centromeric regions, suggesting that PfHP1 does not contribute to the maintenance of centromere structure and function in this organism15–18,20. The subtelomeric and chromosome-internal heterochromatic domains collectively cover over 400 protein-coding genes, most of which belong to *P. falciparum*-specific gene families that encode virulence factors exported to the host erythrocyte15–18. In addition, PfHP1 also binds to a small number of euchromatic loci, including the gene encoding the master transcription factor of sexual differentiation PIA2p-G15,17. Consistent with a role for PfHP1 in heritable gene silencing, almost all PfHP1-associated genes are expressed in a clonally variant manner21. The best-studied example is provided by the var gene family that consists of approximately 60 members, each encoding a variant of the erythrocyte membrane protein 1 (PfEMP1) antigen that is exposed on the surface of infected red blood cells (iRBCs)22–25. The PfEMP1-dependent binding of iRBCs to endothelial cells and uninfected RBCs leads to parasite sequestration in the microvasculature, which strongly contributes to severe disease26,27. Importantly, expression of the var gene family is controlled in a mutually exclusive manner (aka singular gene choice), such that at any given time only a single var gene is transcribed while all other family members are epigenetically silenced in a PfHP1-dependent manner28–30. Switches in var gene transcription then lead to clonal antigenic variation of PfEMP1, allowing the parasite to evade adaptive immune responses and establish chronic infection24,26.

Using an inducible PfHP1 loss-of-function parasite line, where PfHP1 expression levels can be modulated via the FKBP/DP-Shield-1 conditional expression system23,24, we recently identified three important roles for PfHP1 in the biology of blood stage parasites25. First, we found that PfHP1 is essential for the heritable silencing of heterochromatic gene families as PfHP1 depletion resulted in the de-repression of almost all var genes and many other subtelomeric gene families in the progeny. Second, we demonstrated that PfHP1 depletion leads to a 25-fold increase in sexual conversion rates, with over 50% of parasites in the progeny differentiating into gametocytes (which are required for malaria transmission via the mosquito vector). This striking phenotype was linked to de-repression of the *pfap2-g* locus in absence of PfHP1. Third, we showed that the remaining asexual parasites in the PfHP1-depleted progeny failed to enter S-phase, revealing a crucial role for PfHP1 in the control of proliferation31.

Studies in model eukaryotes have shown that HP1 is post-translationally modified, particularly by phosphorylation. Phosphorylation of HP1 regulates various functions in a number of cellular processes in fission yeast and mammals, including heterochromatic gene silencing, mitosis and DNA repair33,34. For instance, casein kinase 2 (CK2)-dependent phosphorylation of serine residues in the N-terminal part of Swi6 is important for transcriptional silencing and the recruitment of the histone deacetylase complex SHREC in *S. pombe*35. Similarly, in mice the N-terminal phosphorylation of HP1α by CK2 is important for targeting HP1 to heterochromatin as well as for chromosomal stability. While a single substitution of serine 14 with alanine (S14A) impaired the binding of HP1α to H3K9me3 and caused diffuse heterochromatic localization, multiple substitutions of clustered serines (S11A to S14A) hampered chromosomal integrity36. In humans, HP1 phosphorylation has been shown to play a role in progression through mitosis. Human HP1α is a substrate of the nuclear Dbf2-related (NDR) kinase; in an NDR-depleted cell line, the lack of HP1α phosphorylation at serine 95 in the hinge domain resulted in chromosome alignment defects, aberrant spindle morphology and a delay in metaphase progression37. Furthermore, HP1 phosphorylation has also been shown to play a role in the DNA damage response in humans. CK2-dependent phosphorylation of HP1α at threonine 51 was shown to modulate the dispersion of HP1 from chromatin, which in turns facilitates histone H2AX phosphorylation and recruitment of downstream regulators involved in repairing chromosomal DNA breaks38.

In contrast to model eukaryotes, the functional role of HP1 phosphorylation in *P. falciparum* and the kinases involved are still unknown. Hence, to begin understanding how PfHP1 function is regulated in *P. falciparum*, we studied PfHP1 phosphorylation using *in vitro* and *in vivo* assays. Liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis of immunoprecipitated native PfHP1 showed that PfHP1 is phosphorylated in its CD and hinge domains. *In vitro* kinase assays revealed that PfHP1 is a substrate of *P. falciparum* CK2 (PfCK2). LC-MS/MS analysis showed that PfCK2 targets three clustered serine residues within the PfHP1 hinge region in *vitro*. By generating conditional PfHP1 phosphomutant cell lines using CRISPR/Cas9-mediated genome editing combined with the DiCre/LoxP system39,40, we found that PfCK2-dependent phosphorylation of PfHP1, and phosphorylation of the PfHP1 hinge domain in general, is dispensable for proper PfHP1 localisation, gene silencing, parasite growth and sexual conversion.

**Results**

**Identification of phosphorylated PfHP1 residues and parasite kinases phosphorylating PfHP1.** Several large-scale phosphoproteomics studies in *P. falciparum* collectively detected 13 phosphorylated residues in PfHP1 (T2, S4, S33, T38, S57, S89, S92, S108, T110, S122, S125, S129, S174)42–47. The Y32 and S136 residues have been identified as additional phosphosites in a recent study investigating native PfHP1 complexes48. To confirm and possibly expand these results, we used LC-MS/MS experiments to map phosphorylated residues in native PfHP1. To this end, we purified PfHP1-GFP by immunoprecipitation (IP) from nuclear extracts prepared from 3D7/HPI1-GFP schizont stage parasites31 in four independent biological replicate experiments (Fig. 1a). LC-MS/MS analysis of the eluted protein samples identified a total of eleven phosphosites in PfHP1 (Fig. 1b and Supplementary Table 1). One of these phosphosites (S206) has not been identified in any of the earlier studies, and five previously mapped phosphosites (T38, S57, S92, S108 and T110) have not been identified here (Fig. 1b). Two of the sites identified in our study are located in the first few residues preceding the CD domain (T2, S4), two are located within the CD domain in a predicted flexible loop (Y32, S33), six are located in the hinge region (S89, S122, S125, S129, S136, S174) and one is located in a predicted loop in the CSD domain (S206) (Fig. 1b).
Several of the identified phosphosites conform to CK2 target sites in view of their richness in acidic residues (e.g. S129; SDEE), while others appear as potential targets of proline-directed kinases such as CDK, GSK3 or MAPK (e.g. S174; ESP), or of basic residues-directed kinases such as AGC kinases (e.g. S89; SIK) (Supplementary Table 1) (see Amanchy and colleagues for a comprehensive list of phosphorylation motifs49). To identify candidate kinases possibly phosphorylating PfHP1, we screened a set of six recombinant functional parasite kinases, namely PfCK250, PfGSK351, PfMAP252, PfNEK253, PfNEK454 and PfPK655, for their ability to phosphorylate recombinant PfHP1 in vitro, which includes the potential candidates PfCK2, PfGSK3 and PfMAP2 predicted by the phosphorylation site motifs outlined above. For this purpose, we used purified recombinant full-length PfHP1 and a truncated PfHP1 polypeptide encompassing the CD domain and hinge region (PfCD.H) (Fig. 2a). To perform the kinase reactions, we used an in vitro assay based on luminescence signal detection (ADP-Glo, Promega).

Among the recombinant parasite kinases screened, PfMAP2, PfPK6, PfNEK2 and PfNEK4 showed little or no activity on PfHP1 (Supplementary Fig. 1). PfNEK4 phosphorylated the control substrate but not the recombinant PfHP1 proteins. PfMAP2, PfPK6 and PfNEK4 all displayed high autophosphorylation activities as shown previously52,53,55 and no increased consumption of ATP was observed in presence of the control substrates or PfHP1, suggesting that these kinases do not target PfHP1; however, we cannot exclude that low-level phosphorylation of PfHP1 may have been masked by the high level of autophosphorylation displayed by these three enzymes. PfGSK3 showed some positive enzymatic activity on PfHP1 but this result was inconclusive due to the high level of autophosphorylation exerted by this kinase both in the ADP-Glo assay as well as in a radioactive kinase activity assay employing γ-P32-ATP (Supplementary Fig. 2). Importantly, however, PICK2 showed remarkable activity in phosphorylating PfHP1 in vitro. In the ADP-Glo assay, PICK2 showed autophosphorylation activity and was able to phosphorylate the control substrate β-casein. In absence of PICK2, PfHP1 and PfCD.H exhibited no signals of phosphorylation activity as expected. In contrast, when PICK2 was added to the PfHP1 and PfCD.H substrates, ADP conversion increased substantially compared to PICK2 alone or PICK2 with β-casein...
Figure 2. PfCK2 phosphorylates PfHP1 \textit{in vitro}. (a) Coomassie-stained SDS-PAGE gel showing the purified recombinant PfHP1 and PfCD.H proteins. (b) \textit{In vitro} ADP-Glo assay results reveal that PfCK2 phosphorylates PfHP1 and PfCD.H \textit{in vitro}. The percentage of ADP converted back into ATP (y-axis) is a surrogate measure for kinase activity (i.e. the relative amount of ATP consumed in the kinase reaction). β-casein was included as a positive control substrate for PfCK2. Recombinant PfHP1 and PfCD.H in absence of PfCK2 were used as negative controls. Values represent the average of two replicate reactions. Error bars represent SD. (c) Cropped sections of a Coomassie-stained SDS-PAGE gel (top) and the corresponding autoradiogram (bottom) of the \textit{in vitro} γ-P\textsuperscript{32}-ATP PfCK2 kinase assay performed with recombinant PfHP1 and PfCD.H substrates. β-casein was used as a positive control substrate. 20 µM TBB was used as a specific inhibitor of PfCK2\textsuperscript{50,56}. The full-size Coomassie-stained gel and autoradiogram are shown in Supplementary Fig. 6.

(Fig. 2b). Consistent results were obtained for PfCK2 using the \textit{in vitro} γ-P\textsuperscript{32}-ATP kinase assay. PfCK2 again showed auto-phosphorylation activity but was clearly capable of phosphorylating β-casein as a positive control and, at substantially higher levels, the PfHP1 and PfCD.H substrates (Fig. 2c). The truncated PfCD.H protein was phosphorylated more efficiently compared to full-length PfHP1, possibly because PfCK2 may have improved access to the target sites in the monomeric recombinant PfCD.H protein compared to the homodimers formed by full-length PfHP1\textsuperscript{15}. To further probe the specificity of the CK2-dependent kinase reaction towards PfHP1 and PfCD.H, 4,5,6,7-tetrabromobenzimidazole (TBB), a selective ATP-competitive inhibitor of CK2 across species\textsuperscript{56}, was added to the kinase reactions. The treatment with 20 µM TBB resulted in a significant drop of phosphorylation signal intensity in the reactions containing the substrates (Fig. 2c). Finally, to identify the residues in PfHP1 targeted by PfCK2 \textit{in vitro}, we performed LC-MS/MS analysis of the ADP-Glo kinase assay reactions. The results revealed that PfCK2 phosphorylated a cluster of three serine residues in the hinge domain (S122, S125 and S129). Three additional predicted CK2 target residues in the PfHP1 N-terminus (T2, S4, S33; see Supplementary Table 1), however, were not detected in their phosphorylated form. As expected, no phosphorylated sites were detected in recombinant PfHP1 prior to the phosphorylation assay (Supplementary Table 2). We also failed to detect any phosphorylated residues in PfHP1 after incubation with PfGSK3 (Supplementary Table 2).

Overall, using three independent approaches, we demonstrate that PfHP1 is phosphorylated at least at eleven residues during \textit{intra-erythrocytic} development, that most of these residues are located either at the N-terminus, in predicted loop regions in the CD and CSD domains or in the hinge domain, and that three residues in the hinge region (S122, S125 and S129) are phosphorylated by PfCK2 \textit{in vitro}.

Generation of conditional PfHP1 phosphomutant parasite lines. To begin addressing the \textit{in vivo} functional significance of PfHP1 phosphorylation we concentrated on the hinge domain for three reasons. First, the hinge domain contains the majority of phosphorylated residues in PfHP1. Second, we identified PfCK2 as a kinase able to phosphorylate residues in the hinge domain \textit{in vitro}. Third, the poor sequence homology between PfHP1 and HP1 orthologs from model eukaryotes precluded the reliable prediction of functionally important phosphosites in PfHP1.

To study the potential role of hinge domain phosphorylation in regulating PfHP1 function, we used two subsequent CRISPR-Cas9-based gene editing steps to engineer parasites that allow for the conditional expression of PfHP1 phosphomutants based on the DiCre-loxP system\textsuperscript{40} (Fig. 3a and Supplementary Figs 3 and 4). In the first step, a sera2 intron:loxP element\textsuperscript{41} was inserted into the 5’ end of the endogenous pphp1 gene to obtain the 3D7/N31DC mother line. PCRs on gDNA and cDNA and Sanger sequencing confirmed the correct editing of the pphp1 locus and the correct splicing of the sera2 intron:loxP element (Supplementary Fig. 4). In the second step, a second sera2 intron:loxP element followed by a recodonsised pphp1 gene fused to \textit{gfp} was placed directly
Figure 3. Generation of DiCre-inducible PfHP1 phosphomutants. (a) Schematics of the CRISPR/Cas9-edited pfhp1 loci (left panel) and corresponding PfHP1 protein products (right panel) expressed in the 3D7/N31DC mother line (top) and the 3D7/HP1–3M, 3D7/HP1-HIM and 3D7/HP1-Control parasites (bottom) before (DMSO) and after (RAP) rapamycin-induced DiCre-dependent excision of the wild type pfhp1 locus. HR, homology regions used for homology-directed repair of Cas9-induced DNA double-strand breaks. Blue arrowheads indicate the position of sera2 intron:loxP elements. Red asterisks indicate STOP codons. Blue asterisks indicate the relative position of serine-to-alanine substitutions in the PfHP1 hinge region in the 3D7/HP1–3M and 3D7/HP1-HIM phosphomutants. Orange and blue boxes represent the wild type and recodonised pfhp1/PfHP1 sequences, respectively. Numbers in the gene and protein schematics refer to nucleotide and amino acid positions, respectively. The black arrowheads indicate the binding sites of the F119 and R157 primers used to confirm correct editing of the pfhp1 locus and efficient DiCre-mediated excision upon rapamycin treatment by PCR on gDNA (see b below). (b) PCR on gDNA confirms the correctly edited pfhp1 loci and efficient excision of the endogenous pfhp1 gene after rapamycin treatment in 3D7/HP1–3M, 3D7/HP1-HIM and 3D7/HP1-Control parasites. Using primers F119 and R157 (see a) the correctly edited pfhp1 locus delivers a PCR product of 3781 bps in the 3D7/HP1–3M (left panel), 3D7/HP1-HIM (middle panel) and 3D7/HP1-Control line (right panel). In the 3D7/N31DC mother line (i.e. prior to the insertion of a recodonised pfhp1-gfp fusion gene) this PCR reaction delivers a 2250 bps fragment (right panel). Correct excision of the endogenous pfhp1 gene in rapamycin-treated 3D7/HP1–3M, 3D7/HP1-HIM and 3D7/HP1-Control parasites results in a decrease of the size of the PCR fragment from 3781 bps to 2964 bps. Primers targeting the cg6 control locus (PF3D7_0709200) have been used as control. RAP, rapamycin. (c) The efficiency of DiCre-mediated excision of the endogenous pfhp1 gene and resulting expression of the recodonised PfHP1-GFP fusion proteins has been quantified by counting the number of GFP-positive parasites in paired control
downstream of the *pfhp1* STOP codon (Fig. 3a and Supplementary Fig. 3). In these parasites, activation of the DiCre recombinase by rapamycin is expected to excise the floxed endogenous *pfhp1* gene and to place a recodonised version encoding a PfHP1-GFP phosphomutant under control of the endogenous promoter (Fig. 3a). We generated two such conditional PfHP1 phosphomutant lines called 3D7/HP1–3M and 3D7/HP1-HIM, where either the three serine residues targeted by PICK2 *in vitro* (S122/125/129 A) or the cluster of seven phosphorylated serine residues in the hinge region (S89/92/122/125/129/136/174 A), respectively, have been substituted by non-phosphorylatable alanines. We also generated a control cell line where rapamycin treatment results in the replacement of the endogenous *pfhp1* with a recodonized wild type *pfhp1-gfp* sequence (3D7/HP1-Control) (Fig. 3a).

**PfHP1 phosphomutants still localize to perinuclear heterochromatin.** In *Drosophila melanogaster* and mice, CK2-dependent phosphorylation of HP1 is required for the correct localization of HP1 to heterochromatin. We therefore tested if the PfHP1 phosphomutants PfHP1–3M and PfHP1-HIM still localize to subtelomeric heterochromatin. Live cell fluorescence imaging in late schizonts (40–48 hpi) after rapamycin treatment showed that in each of the three parasitic lines, exclusion of the endogenous *pfhp1* gene was highly efficient and expression of the recodonised PfHP1-GFP variants was observed in close to 100% of parasites in the populations (Fig. 3c). In contrast, parasites in the DMSO-treated control populations did not express GFP-tagged PfHP1 variants as expected. Consistent with the live fluorescence imaging results, analysis of whole parasite protein lysates by Western Blot showed that the rapamycin-treated parasites exclusively expressed the recodonised PfHP1-GFP fusions, while DMSO-treated control parasites exclusively expressed wild-type untagged PfHP1 (Fig. 3d).

**Phosphorylation of serine residues in the PfHP1 hinge domain is not required for parasite multiplication and plays no obvious role in regulating gene silencing.** In a recent study, we showed that PfHP1 is required for parasite multiplication and for heritable gene silencing. Here, we asked if phosphorylation of serine residues in the PfHP1 hinge domain is required for any of these crucial functions. We first monitored the proliferation rates of the PfHP1 phosphomutants and the control line over three consecutive generations after rapamycin treatment. As shown in Fig. 5a, in each parasite line the multiplication of DMSO- and rapamycin-treated parasites was highly comparable. This result indicates that phosphorylation of the PfHP1 hinge domain is not required for the correct targeting and localization of PfHP1 to heterochromatin.
parasites rather seem to be linked to the DiCre-dependent recombination events at the floxed pfhp1 locus. Whatever the mechanisms underlying this puzzling observation, our data clearly suggest that phosphorylation of the hinge domain plays no important role in regulating pfap2-g silencing. Consistent with this result, preliminary experiments based on indirect immunofluorescence experiments showed similar PfEMP1 expression levels in DMSO- and rapamycin-treated parasites of all three lines, suggesting that hinge domain phosphorylation plays also no major role in PfHP1-dependent silencing of subtelomeric virulence genes.

**Discussion**

Heterochromatin-dependent gene silencing is an important mechanism employed by *P. falciparum* for clonally variant gene expression. PfHP1 is one of the critical factors in this process. In addition, PfHP1 is essential for proliferation of blood stage parasites. Phosphorylation of HP1 has been reported to dynamically regulate the function of this chromatin reader protein in a variety of eukaryotic organisms. Our study described here analysed phosphorylation of PfHP1 and its potential functional role in the biology of *P. falciparum* blood stage parasites.

Using immunoprecipitation of native PfHP1-GFP followed by LC-MS/MS analysis we identified eleven phosphorylated residues in PfHP1 purified from schizont stage parasites. All except one of these sites (S206) have either been identified on multiple tryptic peptides and/or in at least two of the independent IP samples analysed here, or they have been detected in previous studies by high-throughput phosphoproteomics approaches and/or the mass spectrometry-based analysis of PfHP1 complexes. Hence, we assume these residues are truly phosphorylated in intra-erythrocytic parasites. Five additional PfHP1 phosphosites (T38, S57, S92, S108, T110) that were identified in previous phosphoproteomics studies were not detected in our immunoprecipitated PfHP1 samples. This may be explained by the fact that in contrast to these previous studies we didn't enrich for phosphopeptides prior to LC-MS/MS analysis. In such raw tryptic digests the detection of phosphopeptides is often suppressed by the presence of the corresponding non-phosphorylated peptides. Furthermore, phosphosite detection by mass spectrometry is not fully comprehensive and indeed none of the previous phosphoproteomics studies detected all PfHP1 phosphosites simultaneously. Alternatively, it is also likely that differences in parasite stage composition between the samples analysed in the various studies may account for differential phosphosite detection.
By screening a number of recombinant *P. falciparum* kinases using two independent kinase activity assays, we found that PfCK2 and PfGSK3 are able to phosphorylate PfHP1 in vitro. However, for PfGSK3 the phosphorylation signals obtained with the control and PfHP1 substrates were not noticeably higher compared to those obtained from auto-phosphorylation. GSK3 is a serine/threonine protein kinase preferring to catalyse substrates upon prior phosphorylation of a residue nearby the consensus sequence (S*/*T*-*X*-X-X-S/*T*)<sup>60,61</sup>. Therefore, it is likely that GSK3 is only able to efficiently phosphorylate substrates if they have been primed by phosphorylation through another kinase, which was not applied in our in vitro kinase assays. Moreover, although PfGSK3 phosphorylated PfHP1 in the ADP-Glo assay and to some extent also in the radioactive kinase assays, no PfGSK3-dependent phosphosites were detected on the in vitro-reacted PfHP1 substrates by LC-MS/MS analysis. Hence, additional experiments with higher sensitivity will be required to confirm if PfHP1 is indeed phosphorylated by PfGSK3.

Importantly, however, we obtained convincing results showing that PfCK2 phosphorylates PfHP1 at a cluster of three serine residues located in the hinge region (LSLS122*DNS125*sLKKS129*DEE), at least in vitro. Serine 122 and S129 are embedded in a typical CK2 phosphorylation consensus target motif rich in acidic residues (S*/*T*-*D/-E-X-D/E*)<sup>62–64</sup>, whereas S125 lacks the favoured acidic residue at position +1 or +3 as the most crucial specificity determinant of the phosphoacceptor site<sup>65</sup>. However, similar atypical CK2 recognition sites containing only one acidic residue between −1 to +5 have indeed been reported<sup>66</sup>. In our efforts to analyse the role of PfCK2-dependent PfHP1 phosphorylation in vivo, however, we found that substituting the three target residues in the hinge domain with non-phosphorylatable alanines (S122/125/129A; PfHP1–3M) had no observable effect on PfHP1 function; parasites expressing the PfHP1–3M triple phosphomutant displayed no marked defects in PfHP1 localisation, *pfap2-g* silencing or mitotic progression. Strikingly, we even failed to observe any silencing- or growth-related phenotypes in parasites expressing the PfHP1-HIM phosphomutant, where seven serine residues in the hinge region have been replaced with alanines (S89/92/122/125/129/136/174A).

In line with these results, it has been reported that phosphorylation of S93 within the mouse HP1α hinge region or S89/91 within the human HP1β hinge domain does not affect HP1's localization to chromatin<sup>67,69</sup>. In
addition, co-immunoprecipitation of human HP1β with histone 3 revealed only a minor role for S89 phosphorylation in the ability of HP1β to bind H3K9me3 in spite of local conformational changes induced upon phosphorylation72. In another study on Swi6, the HP1 ortholog in S. pombe, mutations of CK2-dependent phosphosites in the hinge and CSD domains (S192/212/220/268/274 A) did also not affect heterochromatic gene silencing68. In other systems, however, essential roles of phosphorylation within the HP1 hinge region have still been reported. In humans, the Aurora A- and NDR1-dependent phosphorylation of serine residues in the hinge region of HP1γ and HP1α, respectively, both play crucial roles in mitotic progression during the G2/M phase68,69. Further, protein kinase A (PKA)-dependent phosphorylation of the hinge domain of a D. melanogaster HP1 variant plays important roles in HP1 dimerisation, protein-protein interaction and binding to H3K9me370.

In conclusion, our study confirms that PfHP1 is phosphorylated during intra-erythrocytic development at multiple residues particularly in the CD and hinge domains. We also show that PfCK2 phosphorylates three clustered serine residues in the PfHP1 hinge region in vitro, but the phosphorylation of these sites is not required for proper PfHP1 function in blood stage parasites. If S122, S125, and S129 are indeed phosphorylated by PfCK2 in vivo and if PfCK2 phosphorylates additional target residues in PfHP1 could be tested in future experiments employing conditional PfCK2 knock-down cell lines65. Surprisingly, we also demonstrate that the simultaneous mutation of seven phosphorylation target residues in the hinge domain has no observable effect on parasite viability. We therefore anticipate that phosphorylation of the PfHP1 hinge region may play an important role elsewhere in the parasite life cycle, for instance during meiosis in the mosquito vector and/or in the replicative phases during male gametogenesis, sporogony or exo-erythrocytic schizogony. While our study does not provide functional insight into the role of PfHP1 hinge domain phosphorylation, the experimental pipeline established in this study provides an elegant approach to interrogate protein function and the role of post-translational protein modifications in the biology of P. falciparum blood stage parasites.

Methods
Parasite culture and transfection. 3D7/HP1-GFP parasites31 were cultured at 5% hematocrit in RPMI-1640 medium supplemented with 25 mM HEPES, 100 mM hypoxanthine, 24 mM sodium bicarbonate and 0.5% Albumax II. The transgenic lines generated in this study were cultured in the same medium supplemented with 2 mM choline to reduce background sexual conversion rates as demonstrated recently72. Parasite cultures were synchronized using 5% sorbitol as described73. Co-transfection of CRISPR/Cas9 and donor plasmids into the DiCre-expressing line 3D7/1G5DiCre48 and selection of transfected populations was performed as described recently48.

Immunoprecipitation of native PfHP1-GFP. Parasite samples were isolated from 30 ml culture of 3D7/HP1-GFP early-to-late schizont stage parasites (5% hematocrit) as described previously68. Nuclear proteins were extracted using extraction buffer (2 M L-arginine, 1.925 M HCl, 50 mM H3PO4, and 10 mM TCEP) for 20 min on ice. The extract was cleared by centrifugation for 20 min at 20'000 g and 4 °C. The supernatant was diluted 1:5 with wash buffer 1 (WB1) (PBS containing additional 324 mM NaCl, 1 M L-proline, 1% octyl β-D-glucopyranoside and 2 mM TCEP) and spun again using the same conditions. Extraction buffer, WB1 and buffers used for nuclear isolation were supplemented with 1x protease inhibitor cocktail (Roche), 5 mM 3-aminoacaproic acid (protease inhibitor), 3 mM sodium butyrate (histone deacetylase inhibitor) and 2 mM NaN3, 2 mM β-glycerophosphate, 4 mM sodium tartrate, 1 mM sodium pyrophosphate and 1 mM activated NaVO3 (phosphatase inhibitors). GFP-Trap_A beads (Chromotek) were equilibrated in WB1, added to the supernatant and rotated for 1 h at room temperature (RT). The beads were washed three times with WB1 and twice using WB2 (PBS containing additional 824 mM NaCl and 0.2 mM TCEP). Proteins were eluted using arginine elution buffer (2 M L-arginine, 50 mM acetic acid and HCl to pH 4). The eluate was neutralized by addition of 0.1 volumes of 1 M tris base. Next, the samples were processed and analyzed using LC-MS/MS as detailed below.

Capillary liquid chromatography-tandem mass spectrometry (LC-MS/MS). For the PfHP1-GFP IP experiments, the neutralised elutions were reduced with 10 mM DTT at 37 °C for 1 hr and alkylated with 50 mM iodo-acetamide for 15 min at RT. Proteins were digested with 250 ng endoproteinase LysC (Wako, Neuss, Germany) for two hours at 37 °C followed by 500 ng trypsin (Worthington, Lakewood, NJ, USA) overnight. The digest was stopped with TFA to 1% final concentration and desalted on a microspin column (The Nest Group, Southborough, MA, USA) according to the manufacturer’s recommendations.

For the ADP-Glo in vitro kinase assay samples, 20 µl of the technical duplicate reactions were pooled, precipitated with 20% trichloroacetic acid (TCA) on ice for 30 min, followed by washing the precipitate with 17% TCA and two acetone (ice-cold) washes before air-drying the pellets. The protein pellets were dissolved in 30 µl 100 mM Tris-HCl (pH 8.0)/6 M urea, reduced and alkylated as above and digested with 250 ng endoproteinase LysC for two hours at 37 °C. The urea concentration was diluted to 2 M with 100 mM Tris-HCl (pH 8.0) and the sample was further digested with 500 ng trypsin overnight at 37 °C. The digest was acidified with 1% TFA and the sample was desalted on a MicroSpin cartridge according to the manufacturer’s recommendations.

The eluted peptides were dried in a SpeedVac and dissolved in 40 µl 0.1% formic acid and analysed by capillary LC-MS/MS using a home-packed separating column (0.075 mm × 25 cm) packed with Reprisol C18 reverse-phase material (2.4 μm particle size, Dr. Maisch, Ammerbuch-Entringen, Germany). The column was connected on line to an Orbitrap Elite FT hybrid instrument (Thermo Scientific, Reinch, Switzerland). The solvents used for peptide separation were 0.1% formic acid in water (solvent A) and 0.1% formic acid and 80% acetonitrile in water (solvent B). 2 µl of peptide digest were injected with a Proxeon nLC capillary pump (Thermo Scientific) set to 0.3 μl/min. A linear gradient from 0 to 40% solvent B in solvent A in 95 min was delivered with the nano pump at a flow rate of 300 nl/min. After 95 min the percentage of solvent B was increased to 75% in ten minutes. The eluting peptides were ionized at 2.5 kV. The mass spectrometer was operated in data-dependent
mode. The precursor scan was done in the Orbitrap set to 60,000 resolution, while the fragment ions were mass analyzed in the LTQ instrument. A top twenty method was run so that the twenty most intense precursors were selected for fragmentation. The MS/MS spectra of the four PfHP1-GFP IP samples were searched against a combined *P. falciparum* ([www.plasmoDB.org](http://www.plasmoDB.org); release 9.3)/human annotated protein database using Proteome Discoverer 2.2 (Thermo Scientific, Reinch, Switzerland) using the two search engines Mascot and SequestHT (Supplementary Table 1). The PfHP1-GFP IP replicate samples 1 and 2 were additionally searched against the PfHP1-GFP sequence (Supplementary Table 1). The PKC2 and PGSK3 ADP-Glo *in vitro* kinase assay samples were searched against the respective PfHP1 and PfCD.H recombinant protein sequences (Supplementary Table 2). For the search, oxidized methionine, N-terminal protein acetylation and phosphorylation on serine, threonine and tyrosine were used as variable modifications. The identifications were filtered for a false discovery rate of 1%.

**Generation of *E. coli* expression vectors.** In order to increase the solubility strength of the SUMO tag, sequences encoding additional solubility tags were inserted upstream of the sequence encoding an N-terminal 6xHis-SUMO tag as suggested elsewhere. For this purpose, the His-SUMO-encoding sequence was PCR amplified from pETA-HS using the primers Bsa_HisF and T7term. The *BsaI*/*Xhol*-digested product was then cloned into *BamHI*/*Xhol*-cut pGB1 (a kind gift of S. Hiller) and pETAB-MBP, yielding the GB1-His-SUMO (pETA-GHS) and MBP-His-SUMO (pETA-MHS) expression vectors, respectively. Two gene fragments were amplified from 3D7 genomic DNA; full-length *php1* (using primers HP1_F and HP1_XhoR) and a truncated version encoding the CD and hinge domains only (PfCD.H: M1-T181 (using primers HP1_F and CDH_XhoR). *Xhol*-digested PCR products were ligated into *SfoI*/*Xhol*-cut expression vectors; full-length *php1* was cloned into pETA-MHS and *pfcd.h* into pETA-GHS. All primer sequences are listed in Supplementary Table 3.

**Expression and purification of recombinant PfHP1.** Both recombinant proteins were expressed in *E. coli* Rosetta2 (DE3) cells (Novagen) using auto-induction at 22 °C in ZYM-5052 medium. Expression cultures were spun down at 4°C and the pellets were kept at −20°C. Both recombinant proteins were purified using nickel affinity (A), followed by dextrin affinity (MHS-HP1 only) (B) and tag removal (C). *PfCD.H* was further purified using hydrophobic interaction chromatography (HIC) (D). Both proteins were polishes using gel filtration (E). All affinity columns used were produced by GE Healthcare. (A) Nickel affinity. *E. coli* pellets were resuspended in buffer N-A (50 mM H3PO4, 20 mM imidazole, 500 mM NaCl, 5 mM EACA and NaOH to pH 7.4) and lysed by sonication. The lysates were loaded on 1 ml HisTrap columns, washed with 20 column volumes (CV) of the same buffer and eluted using N-B (50 mM H3PO4, 225 mM imidazole, 500 mM NaCl, 5 mM EACA). For full length PfHP1, 2 M urea was included in the lysis buffer. The nickel eluate containing *PfCD.H* was buffer exchanged to subtraction buffer (0.75x concentrated N-A complemented with 10% glycerol, 1 mM TCEP and additional 125 mM NaCl) using three 5 ml HiTrap desalting columns. (B) Dextrin affinity (MHS-HP1 only). The protein was eluted from the nickel column directly on a 5 ml MBPTrap HC column placed below the HisTrap column. After elution, the nickel column was removed and the MBPTrap column was washed with 5 CV of N-A and eluted with N-A containing 2 M urea, 10 mM maltose and 1 mM TCEP (C) Tag removal. The GHS and MHS tags were cleaved off using recombinantly expressed SUMO protease (L403-K621 of *S. cerevisiae* ULP1; expressed as GB1-ULP1–6xHis fusion and purified by nickel affinity and gel filtration) in a ratio of 1:200 and incubated for 1.5 hours at 16°C. In order to subtract the tag, the protease and other contaminants, the digest was passed through a HisTrap column. In the case of full-length PfHP1, guanine-HCl was added to a final concentration of 1 M for this step. (D) HIC (PfCD.H only). Ammonium sulfate from a 4 M stock (pH adjusted to 7 using NH4OH) was added to the protein sample to 1.5 M and this mixture was loaded on a 1 ml Phenyl HP column equilibrated in buffer HIC (1.5 M ammonium sulfate, 5 mM EACA, 0.5 mM EDTA, 20 mM H3PO4-KOH, pH 6, and 10% Glycerol). The column was washed with 20 CV of the same buffer and the protein eluted using a 25 CV long linear gradient, from 1.5 M to 0 mM ammonium sulfate. (E) Gel filtration. *PfCD.H* was polished using 10 mM MOPS-KOH, pH 7, 100 mM NaCl, 10% glycerol buffer and a Superdex75 10/300 GL column. For full-length PfHP1, a HiLoad 26/60 Superdex 200 column was prepared in three steps: first, it was equilibrated in storage buffer (20 mM MOPS-KOH, pH 7, 0.5 M NaCl, 10% glycerol). Then, a gradient (1/10th CV) from storage buffer to refolding buffer (20 mM MOPS-KOH, pH 7, 800 mM arginine, 267 mM citric acid) was loaded to the column, and finally a second gradient (1/8th CV), from refolding buffer to buffer N-A containing 2 M urea and 1 M guanine-HCl was loaded. As a result, the protein passed first through refolding and later through storage buffer. Both proteins were concentrated using Amicon spin filter (Millipore) with a 10× cut-off and stored at −80°C. Their purity and concentration were determined by SDS-PAGE and NanoDrop™ 2000/2000c spectrophotometer with UV extinction coefficients calculated by protparam (https://web.expasy.org/protParam/).

**In vitro kinase assays.** Purified recombinant *P. falciparum* kinases were prepared as described for PKC2, PGSK3 and PIMAP2, PINEK2, PINEK4, PIPK6. The ADP-Glo *in vitro* kinase assay was performed in duplicates according to the manufacturer's instructions (Promega, USA). In this assay, the enzymatic reaction starts when a kinase is added into a mixture containing the substrate and ATP. Upon completion of the reaction, the ADP-Glo assay quantifies the levels of ADP released from consumed ATP as a measure of kinase activity. The exact amount of recombinant kinase (0.6–7 μg) and PfHP1 or PfCD.H (1.8–5.8 μg) substrates used in each reaction is indicated in the corresponding figures. 10 μg of either histone from calf thymus, bovine myelin basic protein (MBP) or β-casein from bovine milk (Sigma-Aldrich) were used as positive controls. 5 μl out of 25 μl of the kinase reactions were used for the final ATP depletion and detection steps. The remaining reaction volume was used for LC-MS/MS analysis to identify phosphosites (see above).

The γ-P32-ATP *in vitro* kinase assays were performed in a standard 25 μl reaction in kinase buffer (20 mM Tris HCl pH 7.5, 20 mM MgCl2, 2 mM MnCl2, 10 mM glycerolphosphate and 10 mM NaF) containing 10 μM ATP, 5
μCi γ-P32-ATP (3000 Ci/mmol, Amersham Biosciences), substrates (5.4 μg PfHP1, 5.8 μg PfCD.H, 10 μg β-casein, 10 μg calf thymus histone, or 5 μg MBP) and recombinant kinases (0.5 μg PICK2 or 0.6 μg PfGSK3). In the assay using PICK2, 20 μM of 4,5,6,7-tetramobromobenzimidazole (TBB) in DMSO was used as a specific CK2 inhibitor45,46. The reactions were carried out for 30 min at 30°C and stopped by the addition of Laemmli buffer. The samples were analyzed by SDS-polyacrylamide gel electrophoresis followed by autoradiography.

**Transfection constructs.** We applied CRISPR/Cas9-mediated genome editing and the DiCre/LoxP system4,43 to generate parasite lines for the conditional expression of PfHP1-GFP phosphomutants. We engineered (1) 3D7/N31DC_PfHP1–3M (3D7/HP1–3M) for expression of the S122/125/129A PfHP1 mutant; (2) 3D7/N31DC_PfHP1-HIM (3D7/HP1-HIM) for expression of the S89/92/125/129/136/174A PfHP1 mutant; and (3) 3D7/N31DC_PfHP1-Control (3D7/HP1-Control) for expression of wild type PfHP1-GFP. To obtain these cell lines we performed two subsequent transfection steps.

In the primary transfection, we generated the mother cell line 3D7-1G5DC/S′-loxPInt-g31 (3D7/N31DC), which carries a sera2 intron:loxP element44 inserted into the 5′ end of the pfhp1 coding sequence. To achieve this, we constructed the pHF-gC-guide31 plasmid by inserting two annealed complementary oligonucleotides (F-g31 and R-g31) encoding the sgRNA target sequence and containing appropriate single-stranded overhangs into the BsaI-digested pHF-gC SpCas9 plasmid45 using T4 DNA ligase. The sgRNA target sequence (S′-ATTATTTTAGATAATGGAA-A3′) is positioned at bps +70 to +89 within the pfhp1 coding sequence and was identified using the CHOPCHOP web tool (http://chopchop.cbu.uib.no)78,79. The donor plasmid pFdon-N31 was generated by Gibson assembly joining four PCR fragments encoding (1) the pFdon plasmid backbone43 digested with SalI and EcoRI; (2) the 103 bp fragment encoding the sera2 intron:loxP fragment amplified from pD_SIP2GFP plasmid (L. Niedergerke, unpublished) using primers F159 and R145; (3) a 5′ homology region (5′ HR) spanning bps −490 upstream of the start codon to +87 in the pfhp1 coding sequence amplified from 3D7 gDNA using primers F147 and R145; and (4) a 3′ HR spanning bps +88 to +756 of the pfhp1 coding sequence amplified from 3D7 gDNA using primers F146 and R144 (the pfhp1 coding sequence is 798 bps long). For transfection, 50 μg of each plasmid (pHF-gC-guide31 and pFdon-N31) were mixed and co-electroporated into DiCre-expressing 3D7/1G5DC parasites40. Transfected parasites were selected with 4 nM WR99210 for six days and then cultured in absence of drug selection until transgenic populations were established.

In the second step, 3D7/N31DC parasites were transfected again to generate parasite lines 3D7/HP1-3M, 3D7/HP1-HIM and 3D7/HP1-Control that carry a second sera2 intron:loxP sequence directly downstream of the endogenous pfhp1 STOP codon, followed by a recomdonised mutated (HP1-3M and HP1-HIM) or wild type (HP1-Control) pfhp1-gfp sequence using the following cloning steps. First, we constructed the pBF-gC-guide250 plasmid by inserting two annealed complementary oligonucleotides (F-g250 and R-g250) encoding the pfhp1 coding sequence ending with a STOP codon, followed by a recodonised mutated (HP1-3M and HP1-HIM) or wild type (HP1-Control) pfhp1-gfp sequence using the following cloning steps. The pBF-gC-guide250 plasmid was digested with SalI and EcoRI, and the 103 bp fragment encoding the sera2 intron:loxP fragment (see Supplementary Methods) using primers F91 and R165, the former of which introduces the S125/129A mutation, was amplified from pD_SIP2GFP plasmid (L. Niedergerke, unpublished) using primers F159 and R145. This, we constructed the pHF-gC-guide31 plasmid by inserting two annealed complementary oligonucleotides (F-g31 and R-g31) encoding the sgRNA target sequence and containing appropriate single-stranded overhangs into the BsaI-digested pHF-gC SpCas9 plasmid45 using T4 DNA ligase. The sgRNA target sequence (S′-AAAAATTTAGAGTTCCTG-3′) is positioned at bps +751 to +770 within the pfhp1 coding sequence (negative strand) and was identified using CHOPCHOP (http://chopchop.cbu.uib.no). Second, we constructed the three donor plasmids. The pD-HP1-Control plasmid was constructed by Gibson assembly joining two PCR fragments. The first fragment was amplified from the plasmid pD-PH1-KO (see Supplementary Methods) using primers F162 and R143 and contains, in the following order, the gfp coding sequence ending with a STOP codon, a 3′ HR spanning the 824 bps directly downstream of the pfhp1 STOP codon, the pD-plasmid backbone43, a 5′ HR spanning bps +88 to +798 of the pfhp1 coding sequence carrying eight synonymous mutations between bps +757 to +798 (see Supplementary Methods) and ending with a STOP codon followed by the 103 bp sera2 intron:loxP element. The second PCR fragment was amplified from a plasmid containing a synthetic recodonized pfhp1 sequence (pUC57-re-pfhp1) (GenScript)56 (see Supplementary Fig. 3 and Supplementary Methods) using primers F164 and R165 and spans bps +88 to +798 of the pfhp1 coding sequence omitting the STOP codon.

The pD-HP1–3M plasmid was constructed by Gibson assembly joining four PCR fragments encoding (1) the 5′ HR spanning bps +88 to +798 of the pfhp1 coding sequence ending with a STOP codon followed by the 103 bp sera2 intron:loxP element and bps +88 to +384 of the recomdonised pfhp1 sequence amplified from the pD-PHP1-1-Control plasmid using primers F158 and R168, the latter of which introduces the S122/125A mutations into PHIP1; (2) a fragment spanning bps +367 to +798 of the recomdonised pfhp1 sequence amplified from pUC57-re-pfhp1 (GenScript)56 using primers F91 and R165, the former of which introduces the S125/129A mutations into PHIP1; (3) the gfp coding sequence sequence ending with a STOP codon followed by the 3′ HR amplified from the pD-PHP1-1-Control plasmid using primers F164 and R172, the latter of which introduces the S89/92/136A mutations; (2) bps +261 to +409 of the recomdonised pfhp1 sequence amplified from pD-PHP1-1–3M (containing the S122/125/129A mutations) using primers F171 and R174, the former of which introduces the S89/92/125A mutations and the latter of which introduces the S136A mutation; (3) bps +390 to +535 of the recomdonised pfhp1 sequence amplified from pD-PHP1-1-Control using primers F173 and R176, the former of which introduces the S136A mutation and the latter of which introduces the S174A mutation; and (4) a fragment containing, in the following order, bps +520 to +798 of the recomdonised pfhp1 sequence, the gfp coding sequence sequence ending with a STOP codon and 3′ HR amplified from pD-PHP1-1-Control using primers F175 and R163, the former of which introduces the S174A mutation. Second, the resulting fragment from the first step was used as template for a second round of PCR amplification using primers F164 and R163 and subjected to a second Gibson assembly joining with two other PCR fragments,
live cell fluorescence microscopy was performed as described before81 with minor modifications using Hoechst for 1 hour80. The cultures were then spun down, washed with an equal volume of culture medium, resuspended in culture medium and returned to culture.

### Nucleic acid isolation and diagnostic PCRs and reverse transcription PCRs.

To confirm correct editing of the pfhp1 locus we performed PCRs using the KAPA HiFi HotStart enzyme (Roche Sequencing Store) on gDNA isolated from the transgenic cell lines. To evaluate the excision efficiency after rapamycin treatment, diagnostic PCRs were performed on gDNA isolated 24–36 hours post rapamycin treatment86. To evaluate the splicing efficiency of the sern2 intron:loxP from the pfhp1 open reading frame, total RNA from the 3D7/N31DC mother cell line and the rapamycin-treated phosphomutant cell lines were isolated using Ribozol (Amresco) according to the manufacturer’s instruction. cDNA was then synthesized using oligo(dT) primers (RetroScript, Invitrogen) and PCRs on cDNA were performed using primers F106 and R107 that amplify the entire coding sequence. All transfection plasmids generated in this study have been validated by Sanger sequencing. All transfection plasmids have been designed and Sanger sequencing results analysed using the SnapGene software (from GSL Biotech; available at snapgene.com). All primer sequences used for PCR are listed in Supplementary Table 3.

### Induction of DiCre recombinase-mediated DNA excision by rapamycin treatment.

Parasites were synchronized twice 16 hours apart to obtain an eight-hour growth window (16–24 hpi). After re-invasion parasites were synchronized again at 0–8 hpi (generation 1) and split into two equal populations, one of which was treated with 0.02% v/v of DMSO (negative control) and the other half was treated with 100 nM rapamycin for 1 hour86. The cultures were then spun down, washed with an equal volume of culture medium, resuspended in culture medium and returned to culture.

### SDS-PAGE and immunoblotting.

After DMSO or rapamycin treatment in generation 1, parasites were allowed to complete schizogony and re-invasion. At 16–24 hpi in generation 2, parasites were released from iRBCs by 0.15% saponin/PBS complemented with 1X protease inhibitor (Roche Diagnostics). After washing 2–3 times in ice-cold PBS, parasite pellets were lysed in 70 °C pre-heated Urea/SDS buffer (8 M Urea, 5% SDS, 50 mM Bis-Tris, 2 mM EDTA, 25 mM HCl at pH 6.5 supplemented with 2 mM DTT and 1X protease inhibitor). Whole parasite protein lysates were separated on NuPage 4–12% Bis-Tris gels (Novex) and analyzed by Western blot using mouse mAb α-GFP (Roche Diagnostics #11814460001), 1:1000; rabbit α-PfHP131, 1:5’000; rabbit α-Histone 4 (Abcam ab10158), 1:10’000. The blot shown in Fig. 3d was first probed with α-GFP antibodies, stripped in 2% SDS, 62.5 mM Tris-HCl (pH 6.8), 100 mM β-mercaptoethanol for 30 min at 60 °C, and re-probed with rabbit α-PHP1 and α-Histone 4 antibodies simultaneously.

### Live cell fluorescence imaging.

To quantify the efficiency of pfhp1 excision after rapamycin treatment, live cell fluorescence microscopy was performed as described before89 with minor modifications using Hoechst (Merck) at a final concentration of 5 µg/ml to stain the nuclei. Excision efficiency was determined as the percentage of GFP-positive schizonts at 40–48 hpi in generation 1 (>100 schizonts counted per experiment). Images were taken at 63-fold magnification on a Leica DM 5000B microscope with a Leica DFC 300 FX camera, acquired via the Leica IM 1000 software, processed using ImageJ software (https://imagej.nih.gov/ij). For each experiment, images were acquired and processed with identical settings.

### Parasite multiplication assay.

Parasites were tightly synchronized twice 16 hours apart, split into two equal populations after re-invasion at 0–8 hpi (generation 1), of which one half was treated with DMSO (negative control) and the other half was induced for DiCre recombinase-mediated DNA excision by rapamycin treatment as described above. Giemsa smears were prepared to determine the parasitaemia at 16–24 hpi (generation 1). Giemsa-stained smears were prepared every second day onwards for three generations. Parasitaemia was counted by visual inspection of Giemsa-stained blood smears (>3’000 RBCs counted per experiment). Multiplication rates were determined as the parasitaemia observed in the following generation divided by the parasitaemia observed in the previous generation.

### Gametocyte conversion assay.

Parasites were tightly synchronized twice 16 hours apart and split into two equal populations after re-invasion at 0–8 hpi (generation 1), of which one half was treated with DMSO (negative control) and the other half was induced for DiCre recombinase-mediated DNA excision by rapamycin treatment as described above. At 16–24 hpi in the subsequent generation (day 1 of gametocytogenesis), cultures were treated with 50 mM N-acetyl-D-glucosamine (GlcNAc) for six days to eliminate asexual parasites56,59 and then cultured with normal culture medium for another 4–6 days to observe gametocyte maturation. Gametocytogenesis was determined on day 6 by visual inspection of Giemsa-stained blood smears. Sexual conversion rates were determined as the gametocytogenesis observed on day 6 as a proportion of the total parasitaemia observed on day 1.

### Data availability

All data generated or analysed during this study are included in this published article (and its Supplementary Information files).
References

1. Lachner, M., O’Carroll, D., Rea, S., Mechtler, K. & Jenuwein, T. Methylation of histone H3 lysine 9 creates a binding site for HP1 proteins. *Nature* **410**, 116–120 (2001).

2. Bannister, A. J. et al. Selective recognition of methylated lysine 9 on histone H3 by the HP1 chromo domain. *Nature* **410**, 120–124 (2001).

3. Lomberk, G., Wallrath, L. & Urrutia, R. The Heterochromatin Protein 1 family. *Genome Biol.* **7**, 228 (2006).

4. Grewal, S. I. & Moazed, D. Heterochromatin and epigenetic control of gene expression. *Science* **301**, 798–802 (2003).

5. Kwon, S. H. & Workman, J. L. The heterochromatin protein 1 (HP1) family: put away a bias toward HP1. *Sci. Rep.* **9**, 687–701 (2013).

6. Zeng, W., Ball, A. R. Jr. & Yokomori, K. HP1: heterochromatin binding proteins working the genome. *Epigenetics* **5**, 287–292 (2010).

7. Patero, J. S., Hartnett, T. & Eisenberg, J. C. Functional analysis of the chromo domain of HP1. *EMBO J.* **14**, 3977–3986 (1995).

8. Jacobs, S. A. et al. Specificity of the HP1 chromo domain for the methylated N-terminus of histone H3. *EMBO J.* **20**, 5232–5241 (2001).

9. Cowieson, N. P., Partridge, J. E., Allshire, R. C. & McLaughlin, P. J. Dimerisation of a chromo shadow domain and distinctions from the chromodomain as revealed by structural analysis. *Curr. Biol.* **10**, 517–525 (2000).

10. Brasheer, S. V. et al. The structure of mouse HP1 suggests a unique mode of single peptide recognition by the shadow chromo domain dimer. *EMBO J.* **19**, 1587–1597 (2000).

11. Schotta, G. et al. Central role of Drosophila SU(VAR)3-9 in histone H3-K9 methylation and heterochromatic gene silencing. *EMBO J.* **21**, 1121–1131 (2002).

12. Sugimoto, K., Yamada, T., Muro, Y. & Himeno, M. Human homolog of Drosophila heterochromatin-associated protein 1 (HP1) is a DNA-binding protein which possesses a DNA-binding motif with weak similarity to that of human centromere protein C (CENP-C). *J. Biochem.* **120**, 153–159 (1996).

13. Muchardt, C. et al. Coordinated methyl and RNA binding is required for heterochromatin localization of mammalian HP1alpha. *EMBO Rep.* **3**, 975–981 (2002).

14. Meehan, R. R., Kao, C. F. & Pennings, S. HP1 binding to native chromatin in vitro is determined by the hinge region and not by the chromodomain. *EMBO J.* **22**, 3164–3174 (2003).

15. Fluack, C. et al. Plasmodium falciparum heterochromatin protein 1 marks genomic loci linked to phenotypic variation of exported virulence factors. *PLoS Pathog.* **5**, e1000569 (2009).

16. Salcedo-Amaya, A. M., Hoeijmakers, W. A., Bartfai, R. & Stunnenberg, H. G. Malaria: could its unusual epigenome be the weak link? *PLoS Pathog.* **7**, 51–54 (2011).

17. Scherf, A., Lopez-Rubio, J. & Scherf, A. Genome-wide analysis of heterochromatin associates clonally variant gene regulation with perinuclear repressive centers in malaria parasites. *Cell Host Microbe.* **5**, 179–190 (2009).

18. Perez-Toledo, K. et al. Plasmodium falciparum heterochromatin protein 1 binds to tri-methylated histone 3 lysine 9 and is linked to mutually exclusive expression of var genes. *Nucleic Acids Res.* **37**, 2596–2606 (2009).

19. Hoeijmakers, W. A. et al. Plasmodium falciparum centromeres display a unique epigenetic makeup and cluster prior to and during schizogony. *Cell Microbiol.* **14**, 1391–1401 (2012).

20. Rovira-Graells, N. et al. Transcriptional variation in the malaria parasite Plasmodium falciparum. *Genome Res.* **22**, 925–938 (2012).

21. Baruch, D. I. et al. Cloning the F. falciparum gene encoding PEMP1, a malarial variant antigen and adherence receptor on the surface of parasitized human erythrocytes. *Cell* **82**, 77–87 (1995).

22. Su, X. Z. et al. The large diverse gene family var encodes proteins involved in cytoadherence and antigenic variation of Plasmodium falciparum-infected erythrocytes. *Cell* **82**, 89–100 (1995).

23. Smith, J. D. et al. Switches in expression of Plasmodium falciparum var genes correlate with changes in antigenic and cytoadherent phenotypes of infected erythrocytes. *Cell* **82**, 101–110 (1995).

24. Gardiner, M. J. et al. Genome sequence of the human malaria parasite Plasmodium falciparum. *Nature* **419**, 498–511 (2002).

25. Scherf, A., Lopez-Rubio, J. I. & Riviere, L. Antigenic variation in Plasmodium falciparum. *Annu. Rev. Microbiol.* **62**, 445–470 (2008).

26. Hvid, L., Jensen, A. T. & PIEMPI, A. Parasite Protein Family of Key Importance in Plasmodium falciparum Malaria Immunity and Pathogenesis. *Adv. Parasitol.* **88**, 51–84 (2015).

27. Scherf, A. et al. Antigenic variation in malaria: in situ switching, relaxed and mutually exclusive transcription of var genes during intra-erythrocytic development in Plasmodium falciparum. *Cell* **17**, 5418–5426 (1998).

28. Guizzetti, J. & Scherf, A. Silence, activate, poise and switch! Mechanisms of antigenic variation in Plasmodium falciparum. *Cell Microbiol.* **15**, 718–726 (2013).

29. Deitsch, K. W. & Dzikowski, R. Variant Gene Expression and Antigenic Variation by Malaria Parasites. *Annu. Rev. Microbiol.* **71**, 625–641 (2017).

30. Brancucci, N. M. R. et al. Heterochromatin protein 1 secures survival and transmission of malaria parasites. *Cell Host Microbe* **16**, 165–176 (2014).

31. Banaszynski, L. A., Chen, L. C., Maynard-Smith, L. A., Ooi, A. G. & Wandless, T. J. A rapid, reversible, and tunable method to regulate protein function in living cells using synthetic small molecules. *Cell* **126**, 995–1004 (2006).

32. Armstrong, C. M. & Goldberg, D. E. An FKBP destabilization domain modulates protein levels in Plasmodium falciparum. *Nat. Methods* **4**, 1007–1009 (2007).

33. Kwon, S. H. & Workman, J. L. The changing faces of HP1: From heterochromatin formation and gene silencing to euchromatic gene expression: HP1 acts as a positive regulator of transcription. *Bioessays* **33**, 280–289 (2011).

34. Shimada, A. & Murakami, Y. Dynamic regulation of heterochromatin function via phosphorylation of HP1-family proteins. *Epigenetics* **5**, 30–33 (2010).

35. Shimada, A. et al. Phosphorylation of Swi6/HP1 regulates transcriptional silencing at heterochromatin. *Genes Dev.* **23**, 18–23 (2009).

36. Hiragami-Hamada, K. et al. N-terminal phosphorylation of HP1[alpha] promotes its chromatin binding. *Mol Cell Biol.* **31**, 1186–1200 (2011).

37. Chakraborty, A. & Prasanth, S. G. Phosphorylation-dephosphorylation cycle of HP1alpha governs accurate mitotic progression. *Cell Cycle* **13**, 1663–1670 (2014).

38. Ayoub, N., Leysekharan, A. D., Rernal, J. A. & Venkitaraman, A. R. HP1-beta mobilization promotes chromatin changes that initiate the DNA damage response. *Nature* **453**, 682–686 (2008).

39. Collins, C. R. et al. Robust inducible Cre recombinase activity in the human malaria parasite Plasmodium falciparum enables efficient gene deletion within a single asexual erythrocytic growth cycle. *Mol. Microbiol.* **88**, 687–701 (2013).

40. Jones, M. L. et al. A versatile strategy for rapid conditional genome engineering using loxP sites in a small synthetic intron in Plasmodium falciparum. *Sci. Rep.* **6**, 21800 (2016).
42. Solyakov, L. et al. Global kinomic and phospho-proteomic analyses of the human malaria parasite Plasmodium falciparum. Nat Commun 2, 565 (2011).
43. Treeck, M., Sanders, J. L., Elias, J. E. & Boothroyd, J. C. The phosphoproteomes of Plasmodium falciparum and Toxoplasma gondii reveal unusual adaptations within and beyond the parasites’ boundaries. Cell Host Microbe 10, 410–419 (2011).
44. Lasonder, E. et al. The Plasmodium falciparum schizont phosphoproteome reveals extensive phosphatidylinositol and cAMP-protein kinase A signalling. J. Proteome Res 11, 3323–3337 (2012).
45. Peave, B. N. et al. Global analysis of protein expression and phosphorylation of three stages of Plasmodium falciparum intraerythrocytic development. J Proteome Res 12, 4028–4045 (2013).
46. Lasonder, E., Green, J. L., Grainger, M., Langsley, G. & Holder, A. A. Extensive differential protein phosphorylation as intraerythrocytic Plasmodium falciparum schizonts develop into extracellular invasive merozoites. Proteomics 15, 2716–2729 (2015).
47. Collins, M. O., Wright, J. C., Jones, M., Rayner, J. C. & Choudhary, I. S. Confident and sensitive phosphoproteomics using combinations of collision induced dissociation and electron transfer dissociation. J Proteomics 103, 1–14 (2014).
48. Filarsky, M. et al. GDV1 induces sexual commitment of malaria parasites by antagonizing HP1-dependent gene silencing. Science 359, 1259–1263 (2018).
49. Amanchy, R. et al. A curated compendium of phosphorylation motifs. Nat Biotechnol 25, 285–286 (2007).
50. Holland, Z., Prudent, R., Reiser, J. B., Cochet, C. & Doerig, C. Functional analysis of protein kinase CK2 of the human malaria parasite Plasmodium falciparum. Eukaryot. Cell 8, 388–397 (2009).
51. Drouiche, E. et al. Plasmodium falciparum glycogen synthase kinase-3: molecular model, expression, intracellular localisation and selective inhibitors. Biochim. Biophys. Acta 1697, 181–196 (2004).
52. Dorin, D. et al. An atypical mitogen-activated protein kinase (MAPK) homologue expressed in gametocytes of the human malaria parasite Plasmodium falciparum. Identification of a MAPK signature. J Biol Chem 274, 29912–29920 (1999).
53. Reiniger, L. et al. An essential role for the Plasmodium Nek-2 Nima-related protein kinase in the sexual development of malaria parasites. J Biol Chem 284, 20858–20868 (2009).
54. Reiniger, L. et al. A NIMA-related protein kinase is essential for completion of the sexual cycle of malaria parasites. J Biol Chem 280, 31957–31964 (2005).
55. Brach-Chi-Richard, V. et al. PIPK6, a novel cyclin-dependent kinase/mitogen-activated protein kinase-related kinase from Plasmodium falciparum. Biochem. J. 347(Pt 1), 255–263 (2000).
56. Sarno, S. et al. Selectivity of 4.5,6.7-tetrabromobenzotriazole, an ATP site-directed inhibitor of protein kinase CK2 (casein kinase 2). FEBS Lett 496, 44–48 (2001).
57. Zhao, T. & Eissenberg, J. C. Phosphorylation of heterochromatin protein 1 by casein kinase II is required for efficient heterochromatin binding in Drosophila. J Biol Chem 274, 15095–15100 (1999).
58. Mivelman, Q. L. et al. Improved synchronous production of Plasmodium falciparum gametocytes in vitro. Mol. Biochem. Parasitol 154, 119–123 (2007).
59. Ponnudurai, T., Lensen, A. H., Meis, J. F. & Meuwissen, J. H. Synchronization of Plasmodium falciparum gametocytes using an automated suspension culture system. Parasitology 93(Pt 2), 263–274 (1986).
60. Doble, B. W. & Woodgett, J. R. GSK-3: tricks of the trade for a multi-tasking kinase. Cell 112, 25–37 (2003).
61. Fiol, C. J., Wang, A., Roeske, R. W. & Roach, P. J. Ordered multisite protein phosphorylation. Analysis of glycogen synthase kinase 3 action using model peptide substrates. J Biol Chem 265, 6061–6065 (1990).
62. Pinna, L. A. Protein kinase CK2: a challenge to canons. J Cell Sci 115, 3873–3878 (2002).
63. St-Denis, N. et al. Systematic investigation of hierarchial phosphorylation by protein kinase CK2. J Proteomics 118, 49–62 (2015).
64. Rusin, S. F., Adamo, M. E. & Kettenbach, A. N. Identification of Candidate Casein Kinase 2 Substrates in Mitosis by Quantitative Phosphoproteomics. Frontiers in cell and developmental biology 5, 9 (2017).
65. Meggio, F., Marín, O. & Pinna, L. A. Substrate specificity of protein kinase CK2. Cell Mol Biol Res 40, 401–409 (1994).
66. Bian, Y. et al. Global screening of CK2 kinase substrates by an integrated phosphoproteomics workflow. Scientific reports 3, 3460 (2013).
67. Munari, F., Gajda, M. J., Hiragami-Hamada, K., Fischle, W. & Zweckstetter, M. Characterization of the effects of phosphorylation by CK2 on the structure and binding properties of human HP1beta. FEBS Lett 588, 1094–1099 (2014).
68. Grzenda, A. et al. Functional impact of Aurora A-mediated phosphorylation of HP1gamma at serine 83 during cell cycle progression. Epigenetics & chromatin 6, 21 (2013).
69. Chakraborty, A., Prasanth, K. V. & Prasanth, S. G. Dynamic phosphorylation of HP1alpha regulates mitotic progression in human cells. Nat Commun 5, 3445 (2014).
70. Badugu, R., Yoo, Y., Singh, P. B. & Kellum, R. Mutations in the heterochromatin protein 1 (HP1) hinge domain affect HP1 protein interactions and chromosomal distribution. Chromosoma 113, 370–384 (2005).
71. Tham, W. H. & et al. Plasmodium falciparum Adhesins Play an Essential Role in Signalling and Activation of Invasion into Human Erythrocytes. PLoS Pathog 11, e1005343 (2015).
72. Brancucci, N. M. B. et al. Lysoosphatidylcholine Regulates Sexual Stage Differentiation in the Human Malaria Parasite Plasmodium falciparum. Cell 171, 1532–1544 e1515 (2017).
73. Lambros, C. & Vanderberg, J. P. Synchronization of Plasmodium falciparum erythrocytic stages in culture. J. Parasitol 65, 418–420 (1979).
74. Malakhov, M. P. et al. SUMO fusions and SUMO-specific protease for efficient expression and purification of proteins. J Struct. Funct. Genomics 5, 75–86 (2004).
75. Bertschi, N. L. et al. Malaria parasites possess a telomere repeat-binding protein that shares ancestry with transcription factor IIIA. Nat. Microbiol 2, 17033 (2017).
76. Studier, F. W. Protein production by auto-induction in high density shaking cultures. Protein Expr. Purif 41, 207–234 (2005).
77. Van Voorhis, W. C. et al. Open Source Drug Discovery with the Malaria Box Compound Collection for Neglected Diseases and Beyond. PLoS. Pathog 12, e1005763 (2016).
78. Montague, T. G., Cruz, J. M., Gagnon, J. A., Church, G. M. & Valen, E. CHOPCHOP: a CRISPR/Cas9 and TALEN web tool for genome editing. Nucleic Acids Res 42, W401–W407 (2014).
79. Labun, K., Montague, T. G., Gagnon, J. A., Thyme, S. B. & Valen, E. CHOPCHOP v2: a web tool for the next generation of CRISPR genome engineering. Nucleic Acids Res 44, W272–W276 (2016).
80. Knappe, E., Napiorkowska, M., van Ooij, C. & Holder, A. A. Generating conditional gene knockouts in Plasmodium - a toolkit to produce stable DiCre recombinase-expressing parasite lines using CRISPR/Cas9. Scientific reports 7, 3881 (2017).
81. Witmer, K. et al. Analysis of subtelomeric virulence gene families in Plasmodium falciparum by comparative transcriptional profiling. Mol. Microbiol 84, 243–259 (2012).
82. Drozdetsky, A., Cole, C., Procter, J. & Barton, G. J. Pred4: a protein secondary structure prediction server. Nucleic Acids Res 43, W389–394 (2015).
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Author contributions
H.T.N.B. designed and performed experiments, analysed data, prepared illustrations and wrote the paper. I.N. designed and performed experiments related to recombinant expression of PfHP1. N.M.B.B. performed sexual conversion assays. M.J.B. expressed several recombinant kinases. M.J.B., W.D. and H.T.N.B. performed the in vitro kinase assay. S.M. performed LC-MS/MS experiments. P.J., I.S.L. and C.D. supervised experiments and provided resources and conceptual advice. T.S.V. conceived of the study, designed, supervised, and analysed experiments, provided resources and wrote the paper. All authors contributed to editing of the manuscript.

Competing interests
The authors declare no competing interests.

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