Comparative Heterochromatin Profiling Reveals Conserved and Unique Epigenome Signatures Linked to Adaptation and Development of Malaria Parasites

Graphical Abstract

Highlights
- Multi-gene families are common targets of heterochromatin in malaria parasites
- Conserved heterochromatic genes are rare and tend to have regulatory function
- Heterochromatin is stable during asexual replication but variable between strains
- Gametocyte differentiation is linked to changes in the heterochromatin landscape

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In Brief
Fraschka, Filarsky et al. performed a genome-wide characterization of heterochromatin organization across multiple species, strains, and life cycle stages of malaria parasites. This revealed that heterochromatic gene silencing is a conserved strategy to drive clonal variation of surface antigens and to control life cycle stage transitions and cell differentiation.

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Comparative Heterochromatin Profiling Reveals Conserved and Unique Epigenome Signatures Linked to Adaptation and Development of Malaria Parasites

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SUMMARY

Heterochromatin-dependent gene silencing is central to the adaptation and survival of Plasmodium falciparum malaria parasites, allowing clonally variant gene expression during blood infection in humans. By assessing genome-wide heterochromatin protein 1 (HP1) occupancy, we present a comprehensive analysis of heterochromatin landscapes across different Plasmodium species, strains, and life cycle stages. Common targets of epigenetic silencing include fast-evolving multi-gene families encoding surface antigens and a small set of conserved HP1-associated genes with regulatory potential. Many P. falciparum heterochromatic genes are marked in a strain-specific manner, increasing the parasite’s adaptive capacity. Whereas heterochromatin is strictly maintained during mitotic proliferation of asexual blood stage parasites, substantial heterochromatin reorganization occurs in differentiating gametocytes and appears crucial for the activation of key gametocyte-specific genes and adaptation of erythrocyte remodeling machinery. Collectively, these findings provide a catalog of heterochromatic genes and reveal conserved and specialized features of epigenetic control across the genus Plasmodium.

INTRODUCTION

Malaria is caused by unicellular eukaryotes of the genus Plasmodium that belongs to an ancient group of obligate endoparasites known as Apicomplexa. The Plasmodium genus comprises a few hundred species infecting birds, reptiles, or mammals, and their radiation is estimated to have occurred about 130 million years ago (Perkins, 2014). Members of the Vinckeia subgenus parasitize non-primate mammals, among which rodents and bats are the most abundant. This group includes parasites of rodents such as Plasmodium berghei, Plasmodium yoelii, Plasmodium Chabaudi, and Plasmodium vinckei, which serve as important models to interrogate Plasmodium biology. Parasites belonging to the subgenera Plasmodium and Laverania infect humans or other primates. Five species are known to naturally infect humans, namely Plasmodium vivax, Plasmodium ovale, Plasmodium malariae, Plasmodium knowlesi (all members of the Plasmodium clade), and Plasmodium falciparum (Laverania clade).

Malaria parasites of mammals are transmitted between their intermediate hosts by female Anopheles mosquitoes. Their life cycle is complex, involving several stage transitions and replication phases as well as colonization of different cell types and tissues. In the bloodstream, parasites invade red blood cells (RBCs) and undergo intracellular multiplication via schizogony, which involves progression through the ring and trophozoite stages followed by multiple nuclear divisions before a single cytokinesis event leads to the production of up to 32 merozoites ready to invade other RBCs. Repeated rounds of these cycles...
### A

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**α-PIHP1/DAPI**

### B

**chr 10**

**HP1/Input (log2)**

Scale: 100,000 bp

### C

**P. falciparum** (403 genes)

**P. vivax** (834 genes)

**P. knowlesi** (355 genes)

**P. chabaudi** (369 genes)

**P. berghei** (192 genes)

**P. yoelii** (907 genes)

(legend on next page)
are responsible for all malaria-related morbidity and mortality. For malaria transmission to occur, mosquitoes must ingest male and female gametocytes with their blood meal. These sexual precursors emerge at a low rate from the proliferating pool of blood stage parasites and are essential to complete sexual reproduction and subsequent sporozoite formation in the mosquito vector. Upon injection into the skin through a mosquito bite, sporozoites migrate to the liver, undergo intra-hepatic schizogony, and release over 10,000 merozoites that commence blood stage infection.

Proteins involved in functions at the host-parasite interface have been key to the evolutionary success of malaria parasites (Swapna and Parkinson, 2017). Genes encoding such factors comprise up to 15% of all parasite genes and belong to various dynamically evolving multi-gene families (Reid, 2015). Characteristic features of these gene families are that (1) they primarily encode proteins exported to the RBC; (2) they display high levels of sequence polymorphism between paralogs and across strains, and substantial differences in copy number between species; (3) mostly locate to subtelomeric gene arrays (with the exception of P. knowlesi where they occur throughout the genome); and (4) are often species- or clade-specific (Pain et al., 2008; Reid, 2015). A prime example of species-specific gene families is the 60-member var gene family in P. falciparum. Each var gene encodes a variant of the major surface antigen P. falciparum erythrocyte membrane protein 1 (PfEMP1) that mediates adhesion of infected RBCs (iRBCs) to several host receptors (Smith et al., 2013).

Members of gene families represented in multiple species include the Plasmodium interspersed repeat (pir) genes (Cunningham et al., 2010), fam-a, -b, -c genes (Otto et al., 2014), Plasmodium helical interspersed subtelomeric (phist) genes (Sargeant et al., 2006; Warncke et al., 2016), or reticulocyte-binding-like (rbl) genes (Gunalan et al., 2013). Independent of their size and species distribution, these gene families provide a fertile ground for genetic diversification and are a driving force of evolutionary adaptation.

A number of studies conducted in P. falciparum showed that these multi-gene families are located in heterochromatin (Flueck et al., 2009; Lopez-Rubio et al., 2009; Salcedo-Amaya et al., 2009). Heterochromatin is characterized by trimethylation of lysine 9 on histone H3 (H3K9me3) and the consequent binding of heterochromatin protein 1 (HP1), a conserved regulator of heterochromatin formation and heritable silencing (Lomberk et al., 2006). P. falciparum encodes a single HP1 protein termed PfHP1 (Perez-Toledo et al., 2009; Flueck et al., 2009). In asexual blood stage parasites, PfHP1/H3K9me3 demarcate large heterochromatic domains in all subtelomeric regions and in a few internal regions of some chromosomes (Flueck et al., 2009; Lopez-Rubio et al., 2009; Salcedo-Amaya et al., 2009). These heterochromatic domains are virtually confined to non-syntenic regions and include over 400 genes, almost all of which are members of multi-gene families (Flueck et al., 2009). As a consequence, these genes are subject to clonally variant expression, providing the parasites with a strong potential for phenotypic diversification and rapid adaptation for instance through antigenic variation or expression of alternative invasion ligands or nutrient transporters (Rovira-Graeis et al., 2012; Voss et al., 2014). In addition, a few single genes are also associated with PfHP1, some of which have orthologs in other Plasmodium species (Flueck et al., 2009). One of these loci encodes the transcription factor AP2-G, the master regulator of gametocyto genesis (Kafsack et al., 2014; Sinha et al., 2014). PfHP1-dependent silencing of pFap2-g prevents sexual commitment, while activation of this locus triggers sexual conversion and subsequent gametocyte differentiation, thus facilitating parasite transmission to the mosquito vector (Kafsack et al., 2014; Sinha et al., 2014; Brancucci et al., 2014; Coleman et al., 2014).

These and other studies provided clear evidence that epigenetic regulation, particularly heterochromatin formation, is central to adaptation and survival of malaria parasites. To date, however, heterochromatin organization has almost exclusively been investigated in P. falciparum strain 3D7 blood stage schizonts. It is currently unknown whether the heterochromatin landscape differs between P. falciparum strains, whether other Plasmodium spp. display similar heterochromatin landscapes, or to what extent HP1 contributes to life cycle stage transitions and parasite differentiation.

RESULTS

Conserved and Species-Specific Aspects of the Heterochromatin Landscape across the Plasmodium Genus

To investigate evolutionary aspects of heterochromatin organization, we profiled genome-wide HP1 occupancy in multiple Plasmodium species by chromatin immunoprecipitation sequencing (ChIP-seq). For P. falciparum we used our recently generated polyclonal rabbit α-PfHP1 antibody (Brancucci et al., 2014). Guided by a phylogenetic tree constructed from HP1 orthologs (Figure S1), we generated additional polyclonal rabbit antibodies against PvHP1 (to study HP1 in P. vivax and P. knowlesi) and PbHP1 (to study HP1 in P. berghei, Plasmodium chabaudi, and P. yoelli). Immunofluorescence assays (IFAs) using these antibodies visualized punctate signals in the nuclei of all species that are reminiscent of the perinuclear chromosome end clusters observed in P. falciparum (Brancucci et al., 2014) (Figures 1A and S1). In western blot analyses these antibodies detected a protein of the expected size of HP1 in each species (for P. vivax western blot analysis was not performed due to lack of a suitable parasite sample) (Figure S1).

Figure 1. HP1 Localization and Genome-wide HP1 Occupancy in Six Different Plasmodium Species

(A) IFAs showing HP1 localization (green) in P. falciparum (α-PFHP1 antibodies), P. vivax, and P. knowlesi (α-PvHP1 antibodies), and P. chabaudi, P. berghei, and P. yoelli (α-PbHP1 antibodies) trophozoites. Nuclei were stained with DAPI (blue). Scale bars, 2.5 μm.

(B) Log2-transformed ChIP/input ratio tracks from schizont stages of six Plasmodium species. Coding sequences are shown as blue (sense strand) and red (antisense strand) boxes.

(C) Relative composition of heterochromatic genes in six Plasmodium species, classified into multi-gene families or groups of “unknown,” “unknown exported,” and “other.” Numbers indicate the total number of high-confidence heterochromatic genes. See also Figures S1–S3; Tables S1 and S2.
Using these antibodies we mapped HP1 occupancy in schizonts of *P. falciparum* strain 3D7, *P. berghei* ANKA, *P. chabaudi* chabaudi AS, *P. yoelii* yoelii YM, and *P. knowlesi* clone A1-C.1 (Moon et al., 2013), as well as *P. vivax* field isolates. In all species HP1 predominantly localizes to subtelomeric heterochromatic domains on all chromosomes and to a few internal regions on some chromosomes (Figures 1B and S2). The only exception is *P. knowlesi*, where subtelomeric occupancy is much less pronounced but numerous chromosome-internal HP1-demarcated domains are observed (Figures 1B and S2). We next calculated HP1 enrichment values for each gene and employed a binomial Gaussian mixture model to call HP1-associated genes with high confidence (Figure 1C and Table S1). HP1 occupancy in *P. falciparum* is largely restricted to the var, rif, stever, phist, pfmc-2tm, and other gene families encoding known or predicted exported proteins in accord with a previous report (Flueck et al., 2009). In *P. vivax* most HP1-occupied genes belong to the vir family, and members of the cir, bir, and yir families make up the majority of HP1-associated genes in *P. chabaudi*, *P. berghei*, and *P. yoelii*, respectively. The dispersed HP1-demarcated domains in *P. knowlesi* capture the kir and SICAvar families and the interstitial telomere repeat sequences (ITTs) that are linked to these loci throughout the genome (Pain et al., 2008) (Figures 1C and S3; Table S1). Most other HP1-associated genes in *P. vivax*, *P. knowlesi*, and the three rodent-infecting species are members of gene families encoding other known or predicted exported proteins including *phist*, *stp1*, *fam-a*, *fam-b*, and *fam-c* genes (Figure 1C and Table S1) (Reid, 2015). Moreover, several species possess small heterochromatic gene families involved in RBC invasion such as the *pc235*, *pb235*, and *py235* genes encoding rhoptry proteins (*P. chabaudi*, *P. berghei*, *P. yoelii*) (Iyer et al., 2007), or in metabolism such as *lpf* genes encoding lysophospholipases (*P. falciparum*, *P. vivax*, *P. knowlesi*, *P. chabaudi*, and *P. yoelii*) and *acs* genes encoding acyl-coenzyme A synthetases (*P. falciparum* and *P. chabaudi*).

All species also contain a few HP1-associated genes encoding proteins involved in the regulation of gene expression, vesicular transport, cell division, RBC invasion, and sexual development or transmission (summarized in the category “other”; Figure 1C and Table S1). Notably, while the multi-gene families have no or limited orthology, most of these genes have orthologs including some with conserved synteny across species. The extent of HP1 enrichment at these loci varied across species and most were bound by HP1 only in one species (Tables S1 and S2). However, six conserved syntenic orthologs were associated with HP1 in more than one species (Figure 2A). Four of them encode putative transcriptional or post-transcriptional regulators of gene expression, namely the ApiAP2 TFs AP2-G (Kafsack et al., 2014; Sinha et al., 2014).
and AP2-SP3/AP2-Tel (Modrzynska et al., 2017; Sierra-Miranda et al., 2017), an RNA-binding protein, and a CCCH-type zinc finger (ZnF) protein that is only conserved in P. vivax and P. knowlesi. Interestingly, ap2-g was the only gene with clear HP1 enrichment in all species (Figure 2B), underscoring its crucial role in controlling the switch to sexual differentiation (Kaf-sack et al., 2014; Sinha et al., 2014; Brancucci et al., 2014; Coleman et al., 2014). ap2-sp3/ap2-tel was bound by HP1 in P. vivax and the three species infecting rodents. The two genes encoding the ZnF and RNA-binding proteins and a gene encoding a conserved Plasmodium protein of unknown function were significantly enriched only in the P. vivax/knowlesi clade (Figure 2A), cap380, encoding an oocyst capsule protein essential for oocyst development in P. berghei (Srinivasan et al., 2008), is associated with HP1 in P. vivax, P. knowlesi, P. berghei, and P. chabaudi, and partially marked in P. falciparum, but not in P. yoelii (Figure 2C).

In summary, in all Plasmodium species examined most HP1-enriched genes belong to species-, clade- or pan-specific multi-gene families with documented or probable functions in antigenic variation, immune evasion, or host cell invasion. In addition, each species contains a small number of HP1-associated single-copy genes, many of which are conserved in other Plasmodium spp. and have known or predicted roles in fundamental parasite biology.

**P. knowlesi Parasites Proliferating in Macaque or Human RBCs Display Altered PkHP1 Occupancy at Several Loci**

P. knowlesi parasites have been adapted to continuous in vitro culture in human RBCs (Moon et al., 2013; Lim et al., 2013). We reasoned that the adaptation to growth in human RBCs may have involved epigenetic changes. We therefore compared the PkHP1 binding profiles of P. knowlesi clones A1-C.1 (see above) and A1-H.1, which have been adapted to long-term in vitro culture in Macaca fascicularis and human RBCs, respectively (Moon et al., 2013). Only 12 genes were differentially marked by PkHP1 between the two clones (≥2.5-fold change in PkHP1 occupancy) (Table S3). The six loci with higher PkHP1 enrichment in human RBC-adapted parasites encode a KIR protein, a lysophospholipase, a PHIST protein, and three tryptophan-rich antigens (TRAGs) (Figure 3A and Table S3). The six genes with reduced PkHP1 occupancy in human RBC-adapted parasites encode two members of the SICAvar family, a protein of unknown function, a predicted exported protein, the secreted ookinete protein PSOP7, and a putative histone RNA hairpin-binding protein (Figure 3B and Table S3). Of note, TRAG proteins interact with RBC receptors and have proposed roles in invasion (Tyagi et al., 2015; Zeeshan et al., 2015), and PHIST proteins play central roles in RBC remodeling (Warncke et al., 2016). Furthermore, the P. knowlesi gene encoding a Plasmodium exported protein of unknown function (PKNH_0734900) has an ortholog in P. vivax, a parasite that naturally infects humans. Hence, these epigenetic changes may indeed represent signatures of positive selection during adaptation but replicate in vitro selection experiments, and further characterization of candidates is needed to test this intriguing possibility experimentally.

**Heterochromatin Organization Is Variable between Different P. falciparum Strains**

We next profiled HP1 occupancy in P. falciparum schizont stages of strain NF54 (Delemarre and van der Kaay, 1979), the NF54-derived clone 3D7 (Walliker et al., 1987), and the recently culture-adapted Ghanaian strain P2004 (Elliott et al., 2007; Brancucci et al., 2015) and Cambodian strain NF135 (Teirlick...
**A**

chr 8 (proximal end) scale: 50'000 bp

chr 12 (distal end) scale: 50'000 bp

HP1-ChIP/input (log2)

PI2004

NF54

NF135

3D7

genes

**B**

average HP1-ChIP/input (z-score)

gene clusters 5-11

cluster
genes

13 1
35 2
76 3
22 4
58 5
15 6
20 7
11 8
12 9
7 10
3 11
20 12
1575 13
2444 14
989

PI2004 NF54 NF54 3D7

**C**

genes with variable

HP1 occupancy (clusters 5-11)

(88 genes; 39% pseudogenes)

**D**

genes with invariable

HP1 occupancy (clusters 1-4)

(191 genes; 19% pseudogenes)

*genes in category “other”*

PF3D7_0102500 eba181

PF3D7_0113800 DBL-containing protein

PF3D7_1036300 dblmsp2

PF3D7_1222600 ap2-g

PF3D7_1252400 rh3 (pseudogene)

PF3D7_0102500 eb181

PF3D7_0122200 stevor

PF3D7_013600 DSE-containing protein

PF3D7_014600 ap2-g

PF3D7_1036100 dsmnp2

PF3D7_1036200 pfmc-2tm

PF3D7_1036300 pdbp

PF3D7_1113800 clag3.2

PF3D7_1222600 ap2-g

PF3D7_1252400 rh3 (pseudogene)

PF3D7_0102500 eba181

PF3D7_1220800 unspecified product

PF3D7_0122200 stevor

PF3D7_1222200 unspecified product

PF3D7_1455500 probable protein

*genes in category “other”*

PF3D7_1301200 gplf2

PF3D7_1477800 acbp

PF3D7_1253000 geco

PF3D7_1036400 lsa1

PF3D7_0801300 warp

PF3D7_1475400 crmp4

PF3D7_1475500 ccp1

PF3D7_1252400 rh3 (pseudogene)

PF3D7_0102500 eb181

PF3D7_1476500 probable protein

PF3D7_0302200 clag3.2

PF3D7_0113800 DBL-containing protein

PF3D7_1222600 ap2-g

PF3D7_1036300 dblmsp2

PF3D7_1036300 dblmsp2

PF3D7_1220800 unspecified product

PF3D7_0122200 stevor

PF3D7_1220800 unspecified product

PF3D7_1455500 probable protein

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et al., 2013). To allow direct comparison of PfHP1 occupancy, we mapped all ChiP-seq reads against the 3D7 genome (PlasmoDB v26). The four strains displayed largely similar heterochromatin organization but distinct PfHP1 occupancy was still evident, predominantly at the border of heterochromatic domains (Figure 4A). Importantly, mapping the Pf2004 ChiP-seq reads against the matching Pf2004 genome revealed that many changes in PfHP1 occupancy occurred in syntropic regions (Figure S4), demonstrating that differences at heterochromatin borders are not solely due to genetic rearrangements.

For identification of genes with altered PfHP1 occupancy, ChiP/input enrichment values were Z-score transformed and grouped using k-means clustering (Figure 4B and Table S4). Of all heterochromatic genes (clusters 1–11; 279 genes), one-third displayed variable PfHP1 occupancy across strains (clusters 5–11; 88 genes). Interestingly, most of these genes localize close to heterochromatin boundaries (Figure 4B) and show variation in expression between laboratory lines (Rovira-Graells et al., 2012) (Table S4). While most var, stevor, and rifin genes were stably marked by PfHP1, members of other gene families such as phist, fikk, or surfin and genes encoding unknown exported proteins were over-represented among the variably marked genes (Figures 4C and 4D; Table S4). Pseudogenes were also more abundant in this class, suggesting that they may provide “buffer zones” for heterochromatin reorganization. Variable PfHP1 occupancy was also observed for most PfHP1-associated single-copy genes and small gene families (category “other”) (Figure 4D). This set includes genes encoding proteins implicated in erythrocyte invasion (eba-181) (Gilberger et al., 2003), RBC remodeling in gametocytes (geco) (Morahan et al., 2011), mosquito midgut invasion (warp) (Yuda et al., 2001), sporozoite maturation or egress (ccp1, cmp4) (Simon et al., 2009; Douradinha et al., 2011), or liver stage development (lsa1) (Mikolajczak et al., 2011). Notably, however, four such genes (ap2-g, clag3.2, dblmsp2, and another gene encoding a DBL-domain-containing protein) showed stable PfHP1 enrichment in all strains (Figure 4C), suggesting that stable heterochromatin inheritance at these loci provides a selective growth advantage in vitro. Indeed, depletion of HP1 from the pfp2-g locus leads to cell cycle exit and sexual differentiation (Branucci et al., 2014). dblmsp2 encodes a putative invasion factor expressed only in a small fraction of schizonts (Amamba-Ngwa et al., 2012). clag3.2 and its paralog clag3.1 encode related variants of the surface transport channel PSAC (Nguitragool et al., 2011); parasites express either one of the two variants and some preferentially express clag3.1 in vitro (Cortes et al., 2007; Comeaux et al., 2011). Collectively, these observations highlight a high degree of variability in heterochromatin organization that likely contributes to phenotypic variation of malaria parasites. Interestingly, by analyzing gene expression data from field isolates (Mok et al., 2015) we found that variably marked heterochromatic genes display a significantly higher degree of expression variation compared with euchromatic genes and, to a lesser extent, also to invariably marked genes, suggesting that this relation may be relevant in vivo (Figure S4 and Table S4).

**Heterochromatin Organization Is Invariable between Different Stages of Asexual Intra-erythrocytic Development**

To assess whether and to what extent PfHP1-dependent gene expression contributes to the regulation of gene expression during the intra-erythrocytic developmental cycle (IDC), we mapped PfHP1 occupancy in *P. falciparum* 3D7 ring stages, trophozoites, and schizonts. The profiles were highly similar in all three stages (Figure 5A). PfHP1 enrichment values of individual genes were highly correlated and we did not identify any genes with significantly altered PfHP1 occupancy across the IDC (Figure 5B and Table S5). Comparison of our data with an RNA sequencing (RNA-seq) dataset (Kensche et al., 2016) confirmed that most PfHP1-associated genes are expressed at low levels in the IDC (Flueck et al., 2009) and that most clonally variant genes are PfHP1 target genes (Rovira-Graells et al., 2012) (Figure 5C). Interestingly, genes with lower PfHP1 occupancy showed somewhat higher expression, and this set includes many experimentally confirmed clonally variant genes (Rovira-Graells et al., 2012). var genes appear to be special in this regard since they show moderate expression despite high PfHP1 occupancy levels. In summary, these results reveal that PfHP1-mediated silencing does not contribute in any major way to the temporal regulation of gene expression during the IDC.

**The Switch from Asexual Proliferation to Sexual Differentiation in *P. falciparum* Is Accompanied by Marked Changes in the Heterochromatin Landscape**

We performed PfHP1 ChiP-seq experiments on Pf2004 schizonts, stage II/II gametocytes, and stage IV/V gametocytes. Mapping the PfHP1 ChiP-seq reads against both the 3D7 and Pf2004 reference genomes highlighted clear differences in PfHP1 occupancy between asexual and sexual stages that were particularly evident from the expansion of subtelomeric heterochromatic domains in gametocytes (Figures 6A, 6B, and S5).

Calculation of PfHP1 enrichment values followed by Z-score transformation and k-means clustering identified 104 genes with altered PfHP1 occupancy between schizonts and gametocytes.
Of these, only 15 genes showed reduced PfHP1 occupancy in gametocytes (cluster 5). This set includes pfap2-g (Kafsack et al., 2014), the gametocyte-specific gene pfgeco (Morahan et al., 2011), and seven additional genes at the distal end of chromosome 14 that include five known markers of early gametocytogenesis (pfg14_744, pfg14_748, PF3D7_1476600, PF3D7_1477400, gexp17) (Eksi et al., 2005, 2012; Silvestrini et al., 2010) (Figures 6A, 6B, and S5; Table S6).

Clusters 6–8 contain 89 genes specifically bound by PfHP1 in gametocytes (Figures 6A, 6B, and S6; Table S6). Intriguingly, this set is enriched for genes encoding proteins implicated in RBC remodeling. Of particular interest is the subtelomeric region at the left arm of chromosome 2 where the heterochromatic domain is extended by almost 50 kb in gametocytes. This differentially marked region includes three genes encoding proteins involved in knob formation, namely the knob-associated heat-shock protein 40 (KAHsp40) (Acharya et al., 2012), PfEMP3 (Passaletti et al., 1993), and the knob-associated histidine-rich protein (KAHRP) (Pologe and Ravetch, 1986). In addition, five members of the fikk family, which encode exported serine-threonine protein kinases implicated in host cell remodeling (Nunes et al., 2007; Kats et al., 2014), are enriched in PfHP1 in gametocytes. Increased PfHP1 occupancy is also observed at the gene encoding MESA, an exported protein of unknown function that binds to the RBC membrane skeleton protein 4.1 (Waller et al., 2003) and at 15 phist genes. In summary, these data suggest that heterochromatin remodeling contributes in a major way to the establishment of a gametocyte-specific transcriptional program. It should be noted, however, that the majority of genes differentially expressed between asexual and sexual blood stages are not marked by HP1 in either stage (Young et al., 2005; Flueck et al., 2009), suggesting that sequence-specific transcription factors such as AP2-G and AP2-G2 (Kafsack et al., 2014; Sinha et al., 2014; Yuda et al., 2015) are the main drivers of stage-specific gene expression during sexual differentiation.

Figure 5. Genome-wide PfHP1 Localization Is Invariable across the IDC
(A) Log2-transformed ChIP/Input ratio tracks from P. falciparum 3D7 ring, trophozoite, and schizont stages. Chromosome 3 is depicted as a representative example. Coding sequences are shown as blue (sense strand) and red (antisense strand) boxes.
(B) Pairwise comparisons of PfHP1 coverage of individual genes between the three IDC stages. r, Pearson correlation values.
(C) Scatterplots displaying for each gene the maximum transcript level during the IDC (Kensche et al., 2016) (gray dots) in relation to HP1 occupancy in schizonts (green dots). Genes were sorted according to HP1 occupancy. Genes with clonally variant expression (Rovira-Graells et al., 2012) are marked with a red circle. var genes are indicated as blue dots.

See also Table S5.
Figure 6. Differences in Heterochromatin Organization between Asexual and Sexual *P. falciparum* Blood Stage Parasites

(A) Log$_2$-transformed ChIP-seq ChIP/input ratio tracks from *P. falciparum* Pf2004 schizonts and stage II/III and stage IV/V gametocytes. The proximal end of chromosome 2 is depicted as an example for an expanded heterochromatic domain in gametocytes. Genes involved in knob formation (kahrp, PF3D7_0201900; \( \text{pfemp3} \), PF3D7_1477700, \( \text{pfg14-748, pfg14-744, pf332, gexp17, phist} \)) at the distal end of chromosome 14 have reduced PfHP1 occupancy in gametocytes and are marked in purple.

(B) Heatmap based on k-means clustering of Z-score-transformed ChIP/input ratios calculated for each gene. Examples of genes with reduced (cluster 5) or increased (clusters 6–8) PfHP1 occupancy in gametocytes are highlighted in purple and orange, respectively. Chromosome maps depict the position of genes with reduced (purple) or increased (orange) PfHP1 occupancy in gametocytes in relation to PfHP1-demarcated heterochromatin (green tracks and blue inverted tracks). Genes involved in knob formation (kahrp, \( \text{pfemp3} \), \( \text{pfg14-744, pfg14-744/phist} \), PF3D7_1477400 (phist), PF3D7_1477700 (phist), PF3D7_147700 (pfm14-748/phist), and PF3D7_1478000 (gexp17) at the distal end of chromosome 14 have reduced PfHP1 occupancy in gametocytes and are marked in purple.

See also Figure S5 and Table S6.

DISCUSSION

We show that heterochromatin formation at chromosome ends and their perinuclear clustering is a conserved feature of chromatin organization across the *Plasmodium* genus. Furthermore, in all six species examined heterochromatin primarily embeds members of the various species-, clade-, or pan-specific mutigene families with known or predicted roles in antigenic variation and other host-parasite interrelations, independent of chromosomal location. While in *P. berghei*, *P. chabaudi*, \( \text{P. yoelii} \), and *P. vivax* heterochromatin is mostly confined to chromosome ends, *P. falciparum* features some additional intra-chromosomal heterochromatic islands, and in *P. knowlesi* chromosome-internal HP1-demaracted domains are scattered throughout the genome. These differences in heterochromatin distribution mirror the differences in the genome-wide localization of gene families between the species. These observations, in particular the intriguing association of PfHP1 with the numerous individual kir and SICAvar loci (Figure S3), lends support to the idea that unknown DNA elements linked to sequences of gene family members may be directly involved in the formation and/or local containment of heterochromatin. The ITS elements found at kir
and SICAvar loci are interesting first candidates to be tested for such putative functions.

Some conserved single-copy genes are subject to HP1-dependent gene silencing in at least one of the species analyzed. Heterochromatinization of these genes may be used to prevent their expression during certain phases of the life cycle and/or to express them in a clonally variant manner to facilitate alternative phenotypes conducive to parasite adaptation. Even though some of these genes display only subtle HP1 enrichment and may represent false-positive hits, this list constitutes a valuable resource for the exploration of genus-, clade-, or species-specific heterochromatin genes with functions in key biological processes (Table S2). Here, we focused our attention on the six syntenic orthologs that are heterochromatic in more than one species. Remarkably, next to ap2-g this set includes three other genes with likely roles in regulating gene expression. Of these, AP2-SP3/AP2-Tel has recently been studied in *P. falciparum* and *P. berghei*. In *P. falciparum*, this factor binds the telomeric tract (Sierra-Miranda et al., 2017), and disruption of ap2-sp3/ap2-tel impairs parasite proliferation (Balu et al., 2010). In *P. berghei*, however, AP2-SP3/AP2-Tel is dispensable for intra-erythrocytic growth and sexual development but essential for sporozoite maturation (Modrzynska et al., 2017). These rather conflicting findings may be explained by functional divergence of AP2-SP3/AP2-Tel in different malaria parasites. Consistent with this hypothesis, we found that ap2-sp3/ap2-tel is marked by HP1 in *P. vivax* and the three species infecting rodents, but not in *P. falciparum* and *P. knowlesi*. Another interesting HP1 target gene encodes a putative CCCH-type ZnF protein in *P. vivax* (PVP01_0604500) and *P. knowlesi* (PKNH_0603500). Proteins carrying these domains typically bind RNA and control gene expression by regulating mRNA turnover (Fu and Blackshear, 2017). Hence, this factor may act in a similar way to regulate important processes specifically in the *P. vivax*/*P. knowlesi* clade, and it will be interesting to find out in which life cycle stage(s) this may take effect.

A previous study reported a substantial degree of variegated gene expression in *P. falciparum* and showed that most genes affected are located in heterochromatin (Rovira-Graells et al., 2012). Their results also suggested that the transcriptional states of individual genes are stably inherited during *in vitro* culture. Our results are consistent with these findings. First, up to one-third of all heterochromatic genes showed variable HP1 occupancy between strains, which likely contributes to differential gene expression. Second, we observed a complete lack of variation in HP1 occupancy between the different IDC stages and found only 12 differentially marked genes between two clones of *P. knowlesi* that have been cultured independently in RBCs from two different hosts for over 200 generations (Moon et al., 2013). Together, these findings suggest that heterochromatin is faithfully maintained and that heritable changes occur rather infrequently during asexual proliferation *in vitro*.

In many multicellular eukaryotes, epigenetic mechanisms are employed in a developmental context to progressively silence groups of genes no longer required in differentiated cells (Becker et al., 2016). We found that in a somewhat analogous fashion, many genes display altered HP1 occupancy between asexually reproducing and sexually differentiating parasites. Importantly, since these cell populations were generated from the same strain in one continuous *in vitro* culture experiment, the observed changes directly reflect the dynamics of heterochromatin restructuring associated with the cell fate switch. Besides ap2-g, a few other early gametocyte-specific genes already showed reduced HP1 occupancy in stage II/III gametocytes, suggesting that their derepression occurred alongside that of ap2-g during sexual commitment or in the subsequent gametocyte ring stages. On the contrary, a larger group of genes devoid of HP1 in schizonts became heterochromatinized during gametocyte differentiation. Strikingly, many of these genes encode RBC remodeling factors. The most compelling example is related to the knob structures, parasite-induced aggregates underneath the erythrocyte membrane that are crucial for the PfEMP1-dependent adherence of IRBCs to endothelial cells and their consequent sequestration in the microvasculature (Boddey and Cowman, 2013). Although stage I to IV gametocytes also sequester, primarily in the bone marrow (Joice et al., 2014), their cytoadhesive properties are markedly different and reflected in the absence of knobs in gametocyte-infected erythrocytes (Sinden, 1982; Tiburcio et al., 2013). Our findings suggest that the mechanism responsible for preventing expression of these structures in gametocytes is based on HP1-dependent silencing of kahrp and other genes linked to knob formation. Given that many additional genes implicated in host cell remodeling also become associated with HP1 in gametocytes, we speculate that *P. falciparum* gametocytes use heterochromatin spreading as a general mechanism to inactivate host cell remodeling machinery that is crucial for the survival of asexual parasites but incompatible with the distinct biology of differentiating gametocytes.

Qualitative comparison between our data and genome-wide H3K9me3 ChiP-seq profiles obtained from *P. falciparum* oocysts and salivary gland sporozoites (Gomez-Diaz et al., 2017) suggests that further expansion of heterochromatic domains in these life cycle stages might lead to silencing of yet another set of genes during development in the mosquito (Figure S5). Collectively, our findings reveal that distinct changes in heterochromatin organization accompany developmental stage transitions during parasite transmission, reflecting the different biology, environmental niches, and requirements for rapid adaptive responses associated with each life cycle stage. Such silencing mechanisms must be reversed at some point to enable re-expression of affected genes during the life cycle stages wherein their expression is required. Two recent studies provided evidence that epigenetic reprogramming during mosquito passage may reset virulence gene expression in *P. chabaudi* (Brugat et al., 2017; Spence et al., 2013). Based on our results, HP1 likely plays a central role in such a process, and it will be interesting to build on the tools and knowledge generated here to investigate this intriguing possibility in more detail.

In conclusion, we demonstrate that the HP1-dependent silencing of genes implicated in antigenic variation, invasion, or sexual conversion is evolutionarily conserved in malaria parasites. We further identify a number of genes that are marked by HP1 specifically in one or a few species only. These may play crucial roles in the adaptive control of species- or clade-specific processes. Our results also reveal that gametocyte differentiation is accompanied by changes in heterochromatin distribution that potentially affect the expression of more than 100 genes.
This raises the exciting possibility that despite their large evolutionary distance, malaria parasites employ a strategy similar to that of metazoans to regulate expression of cell-type-specific genes via heterochromatinization.

STAR METHODS

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SUPPLEMENTAL INFORMATION

Supplemental Information includes five figures and seven tables and can be found with this article online at https://doi.org/10.1016/j.chom.2018.01.008.

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AUTHOR CONTRIBUTIONS

S.A.F. and M.F. designed and performed experiments, analyzed data, prepared illustrations, and wrote the paper. I.N. produced affinity-purified *α*-PbHP1 and *α*-PvHP1 antibodies. N.M.B.B. produced crosslinked Pf2004 samples and M.M. supervised these experiments. X.Y.Y., A.T.M., and X.H. produced crosslinked *P. yoelii*, *P. chabaudi*, and *P. berghei* samples and carried out IFAs and western blots. R.H. carried out data analysis, prepared illustrations, and wrote parts of the paper. B.R. and P.R.C. conducted experimental work related to the collection and processing of *P. vivax* blood samples. F.N. provided the diagnostic, clinical, and ethical oversight for the collection of *P. vivax* from malaria patients. R.W.M. and F.M. produced cross-linked *P. knowlesi* samples and carried out IFAs and western blots. Z.B. provided conceptual advice. P.R.P. designed experiments, analyzed data, and wrote parts of the manuscript. R.B. and T.S.V. conceived and supervised this study, analyzed data, prepared illustrations, and wrote the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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Heterochromatin protein 1 secures survival and transmission of malaria parasites. Cell Host Microbe 16, 165–176.


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# STAR Methods

## Key Resources Table

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### Experimental Models: Organisms/Strains

| Mouse: BALB/c (female) | In Vivos Pte Ltd. Singapore | NA |

### Oligonucleotides

| Pb_F: aaaaagtctatcagcagatcagtg | This paper | N/A |
| Pb_R: ttccctcgagccacgttctatatctac | This paper | N/A |
| SUMO_F: ttctatcgactatcactatcagcggctgactcag | This paper | N/A |
| SUMO_R: cctaggatcgcggccaccaatactgtctctgtg | This paper | N/A |
| Pv_F: actgattcggtagaaggtttgaaaatagg | This paper | N/A |
| Pv_R: tgtgtcgtactaggcgtcgggtatcg | This paper | N/A |

### Recombinant DNA

| pET20b(+) | EMD Millipore | Cat#69739-3 |
| pET_PbHP1-6xHis | This paper | N/A |
| pETA-HS | This paper | N/A |
| pETA-HS-PvHP1 | This paper | N/A |
| pETA-Strep | This paper | N/A |
| pETA_Strep-PvHP1-6xHis | This paper | N/A |

### Software and Algorithms

| Clustal Omega | Sievers et al., 2011 | https://www.ebi.ac.uk/Tools/msa/clustalo/ |
| MEGA7 | Kumar et al., 2016 | http://www.megasoftware.net/download_form |
| Olympus DP manager software (v2.2.1.195) | Olympus | N/A |
| ImageJ | Schneider et al., 2012 | https://imagej.net/Downloads |
| Nikon Elements Advanced Research | Nikon | N/A |
| Leica IM1000 software | Leica | N/A |
| Fiji | Schindelin et al., 2012 | https://imagej.net/Fiji |
| SAMtools (v1.2) | Li et al., 2009 | http://www.htslib.org/download/ |
| BEDTools (v2.20.1) | Quinlan and Hall, 2010 | http://bedtools.readthedocs.io/en/latest/content/installation.html |
| UCSC Genome Browser | UCSC Genome Browser | https://genome-store.ucsc.edu/ |
| Rstudio (v3.3.2) | RStudio | https://www.rstudio.com/products/rstudio/download/ |

### Other

| Plasmodipure filters | EuroProxima | Cat#8011Filter2Su |
| HisTrap HP | GE Healthcare | Cat#17-5248-01 |
| HiTrap Protein A HP | GE Healthcare | Cat#17-0403-01 |
| HiTrap NHS activated HP column | GE Healthcare | Cat#17-0716-01 |
| StrepTrap HP | GE Healthcare | Cat#28-9136-30 |
| Amicon Ultra Centrifugal Filter 10KDa | EMD Millipore | Cat#UFC801024 |
| E-Gel Size Select agarose gel | Thermo Fisher Scientific | Cat#6661012 |
| Non-woven fabric filters | ZXBio.net | N/A |

(Continued on next page)
CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for reagents should be directed to and will be fulfilled by the Lead Contact, Till S. Voss (till.voss@swisstph.ch).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Mouse Model

Mice used in this study (BALB/c mice; age 6-8 weeks; weight 25-30 g) were maintained in accordance with the NAACLAR (National Advisory Committee for Laboratory Animal Research) guidelines under the Animal & Birds (Care and Use of Animals for Scientific Purposes) Rules of Singapore with approval from the Institutional Animal Care and Use Committee (IACUC) of Nanyang Technological University (NTU) of Singapore (Approval number: ARFSBS/NIE A002). All animals used in this study were obtained from InVivos Pte Ltd and subsequently housed under SPF conditions at NTU. P. berghei, P. chabaudi and P. yoelii infections for obtaining parasites for chromatin extraction were performed on female BALB/c mice (age 6-8 weeks; weight 25-30g). Mice were infected with an initial inoculum of 5x10^5 parasites and were exsanguinated by cardiac puncture when parasitaemia levels reached between 10-20%.

P. falciparum Parasites

P. falciparum parasites were cultured at 37°C at 5% haematocrit based on the original protocol published by Trager and Jensen (Trager and Jensen, 1978). Growth synchronization was achieved by repeated sorbitol treatments (Lambros and Vanderberg, 1979). 3D7 parasites were cultivated with AB+ human RBCs in RPMI 1640/25 mM Hapes standard culture medium supplemented with 0.5% Albumax II. NF54 and NF135 parasites were cultivated with O+ human RBCs in RPMI 1640/25 mM Hapes standard culture medium supplemented with 10% human serum. P12004/164tdTom parasites (Brancucci et al., 2015) were cultivated with AB+ human RBCs in RPMI 1640/25 mM Hapes standard culture medium supplemented with 10% human serum. P12004/164tdTom gametocytes were generated by inducing sexual commitment as described (Brancucci et al., 2015). After re-invasion cultures were treated with 50mM N-acetylglucosamine (Fivelman et al., 2007) for three consecutive days to eliminate asexual parasites.

P. knowlesi Parasites

P. knowlesi A1-C.1 parasites were grown at 37°C in O+ human RBCs obtained from the United Kingdom National Blood Transfusion Service. P. knowlesi A1-C.1 parasites were grown in M. fascicularis blood provided by NIBSC (UK), which was collected by venous puncture into K2 EDTA BD Vacutainers (Fisher Scientific) as described previously (Moon et al., 2013). Samples of M. fascicularis blood used for parasite culture were provided by the National Institute for Biological Standards and Control. The rationale and procedures for venepuncture and blood sample collection were reviewed by the local Animal Welfare and Ethical Review Body (the Institutional Review Board) of the National Institute for Biological Standards and Control and performed under licence (PPL70/8506) granted by the United Kingdom Home Office as governed by United Kingdom law under the Animals (Scientific Procedures) Act 1986. Animals were handled in strict accordance with the “Code of Practice Part 1 for the housing and care of animals (21/03/05)” available at https://www.gov.uk/research-and-testing-using-animals. The work also met the National Centre for the Replacement Refinement and Reduction of Animals in Research (NC3Rs) guidelines on primate accommodation, care, and use (https://www.nc3rs.org.uk/non-human-primate-accommodation-care-and-use), which exceed the legal minimum standards required by the United Kingdom Animals (Scientific Procedures) Act 1986, associated Codes of Practice, and the US Institute for Laboratory Animal Research Guide. Parasite cultures were synchronised by centrifugation through a density cushion of Nycodenz (Axis-Shield) as previously described (Moon et al., 2013).

P. vivax Parasites

The clinical P. vivax isolates examined in this study were collected under the following approved ethical guidelines and protocols: OXTREC 45-09 and OXTREC 17-11 (Centre for Clinical Vaccinology and Tropical Medicine, University of Oxford, Oxford, UK), MUTM 2008-215 from the Ethics committee of the Faculty of Tropical Medicine (Mahidol University, Bangkok, Thailand) and MRAC No. 16.01 from the Medical Research Advisory Committee of Papua New Guinea. To obtain P. vivax parasites for chromatin extraction eight clinical isolates were collected from malaria patients attending clinics run by the Shoklo Malaria Research Unit, Mae Sot, Thailand, as previously described (Brancucci et al., 2015).
and passed through a Plasmodipure filter (EuroProxima) to remove white blood cells. The purified RBCs were collected by centrifugation through a 0.25 M sucrose cushion (in CLB2) at 2000 rpm for 10 min at 4°C. Crosslinking reactions were quenched by 0.125 M glycine. Nuclei were isolated by releasing parasites from iRBCs using 0.05% saponin followed by lysis in CLB. Nuclei were washed and snap-frozen in CLB supplemented with 50% glycerol. Preparation of sheared chromatin was performed as described above for P. falciparum.

P. knowlesi Sample Collection and Chromatin Preparation

Cultures containing a schizont parasitaemia of around 5% were passed through Plasmodipure filters (EuroProxima) to remove white blood cells prior to formaldehyde crosslinking for 10 min at 37°C and quenching in 0.125 M glycine. Nuclei were isolated by releasing parasites from iRBCs using 0.05% saponin followed by gentle homogenisation (pestle B, 15 strokes) in CLB2 (10 mM Tris–HCl, 3 mM MgCl₂, 0.2% NP40, 1x protease inhibitor (Sigma-Aldrich), pH 8.0) and centrifugation through a 0.25 M sucrose cushion (in CLB2) at 2000 rpm for 10 min at 4°C. Nuclei were snap-frozen in CLB2 supplemented with 20% glycerol. Frozen nuclei were thawed and resuspended in sonication buffer (50mM Tris-HCl, 1% SDS, 10mM EDTA, 1x protease inhibitor (Sigma-Aldrich), pH 8.0) and sonicated for 20-24 cycles of 30 sec ON/30 sec OFF (setting high, Bioruptor™ Next Gen, Diagenode). Chromatin fragment sizes ranged from 100-600 bp as determined by de-crosslinking a 50 µl aliquot and running the purified DNA on a 1% agarose gel.

P. berghei, P. chabaudi and P. yoelii Sample Collection and Chromatin Preparation

For each of the three parasite species, whole blood of infected mice containing approximately 5x10⁹ schizonts (three mice for P. yoelii YM, four mice for P. chabaudi chabaudi AS, five mice for P. berghei ANKA) was diluted in standard P. falciparum culture medium and passed through a Plasmodipure filter (EuroProxima) to remove white blood cells. The purified RBCs were collected by centrifugation at 2’000 rpm for 5 min, resuspended in 30 ml culture medium and crosslinked at 37°C for 10 min in presence of 1% formaldehyde (Sigma-Aldrich). Crosslinking reactions were quenched by 0.125 M glycine. The crosslinked RBCs suspension was split into three equal aliquots, centrifuged at 2’000 rpm for 5 min, supernatants were removed and the RBC pellets snap-frozen in liquid nitrogen. Nuclei were isolated by releasing parasites from iRBCs using 0.05% saponin followed by lysis in CLB (20 mM Hepes, 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 0.65% NP-40, 1mM DTT, 1x protease inhibitor (Sigma-Aldrich), pH 7.9). Again, nuclei were washed and aliquots corresponding to approximately 1x10⁹ nuclei were snap-frozen in CLB supplemented with 50% glycerol. Preparation of sheared chromatin was performed as described above for P. falciparum.

P. vivax Sample Collection and Chromatin Preparation

The P. vivax ex vivo schizont cultures were crosslinked at 37°C for 10 min in presence of 1% formaldehyde (Sigma-Aldrich) and subsequently the reactions were quenched by 0.125 M glycine. The crosslinked RBCs were centrifuged at 200 g for 5 min, supernatants were removed and the RBC pellets snap-frozen in liquid nitrogen. The eight samples were thawed and pooled and nuclei isolated by releasing parasites from iRBCs using 0.05% saponin followed by lysis in CLB. Nuclei were washed and snap-frozen in CLB supplemented with 50% glycerol. Preparation of sheared chromatin was performed as described above for P. falciparum.

Phylogenetic Analysis of Plasmodium HP1 Orthologs

Protein sequences of Plasmodium HP1 orthologs were downloaded from PlasmoDB v33 and used to perform a multiple sequence alignment using Clustal Omega (Sievers et al., 2011) with default parameters. Phylogenetic tree construction was done with MEGA7 (Kumar et al., 2016) using the Neighbor-joining method and 1’000 bootstrap replicates.
Generation and Affinity Purification of α-PbHP1 and α-PvHP1 Antibodies

All recombinant proteins were expressed in Rosetta2(DE3) cells (EMD Millipore) using auto-induction (Studier, 2005). The sequence encoding PbHP1 was amplified from gDNA using primers Pb_F (aaaagatttcatatgacaggatcagatg) and Pb_R (ttccctcgacggctgcagtcgaacct) and cloned into pET20b(+) (EMD Millipore) using NdeI and XhoI restriction sites in order to express PbHP1 fused to a C-terminal 6xHis tag (pET_PbHP1-6xHis). Recombinant PbHP1-6xHis was purified using a HisTrap HP column (GE Healthcare) using buffer NiB (50 mM H2PO4, 0.5 M NaCl, 20 mM imidazole, pH 7.4) supplemented with 8 M urea for lysis, binding and washing, and buffer NIE (50 mM H2PO4, 0.5 M NaCl, 225 mM imidazole, pH 7.4) containing 8 M urea for elution. The elution was diluted 1:4 with H2O and the eluted proteins were precipitated with trichloroacetic acid (TCA). PvHP1 was expressed as an N-terminally tagged 6xHis-SUMO fusion protein (His-PvHP1). The parental expression vector pETA-HS was generated by introducing a sequence encoding a fusion tag consisting of a 6xHis stretch followed by Saccharomyces cerevisiae SUMO, amplified from gDNA using primers SUMO_F (ttcattgcatcatcataactacgagctgacgtaaaga) and SUMO_R (ctttagatctgactcagtgctgctgctg) between the NdeI and BamHI sites of pET20(b)+ (EMD Millipore), yielding a vector similar to the one described by Malakhov and colleagues (Malakhov et al., 2004). The vector was cloned into pET-PbHP1-6xHis and anti-PbHP1 antibodies were generated as previously described for α-PbHP1 antibodies (Fracchia et al., 2018) with the exception that the PbHP1-6xHis-antigen was bound to the nickel column in buffer NiB containing 2 M urea. For the PvHP1 antigen, we first generated the parental pETA_Strep vector facilitating expression of N-terminally Strep(II)-tagged and C-terminally 6xHis-tagged fusion proteins by replacing the NdeI/BamHI fragment in pET20b(+) with an annealed double-stranded oligonucleotide (Strep_F (taaggctacggagaccccggcagctgctg) and Strep_R (gatctcttttcgaatgctgggtctgctgctg)) encoding Met-Ala-Ser-Strep(II). Next, the same PCR product that was used to generate HS_PvHP1 (see above) was cloned into the pETA_Strep vector using BamHI and XhoI to obtain pETA_Strep-PvHP1-6xHis. The Strep(II)-PvHP1-6xHis fusion protein was purified using a HisTrap HP column (GE Healthcare) and washed with five column volumes (CVs) of buffer IgG. Antibodies were eluted in the presence of a linear gradient (8 CVs) of buffers IgGA to IgGB (2 M L-arginine, 150 mM Na2SO4, 150 mM citric acid, adjusted to pH 3.7 using HCl). The antibodies eluted in a symmetrical peak (maximum at 1.4 M arginine and pH 5). Purified antibodies were subject to buffer exchange with PBS.

Affinity purification of α-PbHP1 antibodies was done as previously described for α-PHP1 antibodies (Brancucci et al., 2014) with the exception that the PbHP1-6xHis-antigen was bound to the nickel column in buffer NiB containing 2 M urea. For the PbHP1 antigen, we first generated the parental pETA_Strep vector facilitating expression of N-terminally Strep(II)-tagged and C-terminally 6xHis-tagged fusion proteins by replacing the NdeI/BamHI fragment in pET20b(+) with an annealed double-stranded oligonucleotide (Strep_F (taaggctacggagaccccggcagctgctg) and Strep_R (gatctcttttcgaatgctgggtctgctgctg)) encoding Met-Ala-Ser-Strep(II). Next, the same PCR product that was used to generate HS-PvHP1 (see above) was cloned into the pETA_Strep vector using BamHI and XhoI to obtain pETA_Strep-PvHP1-6xHis. The Strep(II)-PvHP1-6xHis fusion protein was purified using a HisTrap HP column (GE Healthcare) and washed with five column volumes (CVs) of buffer IgG. Antibodies were eluted in the presence of a linear gradient (8 CVs) of buffers IgGA to IgGB (2 M L-arginine, 150 mM Na2SO4, 150 mM citric acid, adjusted to pH 3.7 using HCl). The antibodies eluted in a symmetrical peak (maximum at 1.4 M arginine and pH 5). Purified antibodies were subject to buffer exchange with PBS.

Fluorescence Microscopy

IFAs were performed as described previously (Brancucci et al., 2014). IFAs for P. berghei, P. chabaudi and P. yoelii were performed with acetone:methanol (9:1)-fixed cells using rabbit α-PbHP1 (1:250) and Alexa Fluor 488-conjugated α-rabbit IgG (1:500) (ImmonoJackson). Slides were viewed using a Nikon Ti E inverted microscope using a 100X oil immersion objective and equipped with an Olympus DP30BW camera. Images were acquired with the Olympus DP manager software (v2.2.1.195) and processed using ImageJ (v1.440) (Schneider et al., 2012). IFAs for P. knowlesi were performed using blood smears fixed with 4% paraformaldehyde for 30 min followed by three washes in PBS and permeabilisation in 0.1% Triton-X100 for 10 min. Slides were blocked overnight at 4°C in 3% BSA/PBS and then labelled with rabbit α-PvHP1 (1:600) and Alexa Fluor 488-conjugated α-rabbit IgG (1:5’000) (Thermo Fisher Scientific). The smear was mounted in ProLong Antifade mountant with DAPI (Thermo Fisher Scientific). Slides were viewed using a Nikon Ti E inverted microscope using a 100X oil immersion objective and imaged with an ORCA Flash 4.0 CMOS camera (Hamamatsu). Images were acquired and processed using the Nikon Elements Advanced Research software package. IFAs for P. vivax were performed using methanol-fixed thin blood smears. Slides were blocked using 3% BSA/PBS and then labelled with α-PvHP1 (1:500) and Alexa Fluor 488- conjugated α-rabbit IgG (1:500) (Thermo Fisher Scientific) antibodies in 3% BSA/PBS. Slides were mounted using VECTASHIELD mounting medium containing DAPI (Vector Laboratories). Images were taken
at 100-fold magnification on a Leica DM 5000B microscope with a Leica DFC 345 FX camera, acquired via the Leica IM1000 software, and processed using Fiji (Schindelin et al., 2012). For each experiment, images were acquired and processed with identical settings.

**Western Blot**

*P. berghei*, *P. chabaudi* and *P. yoelii* schizonts were enriched using a 50-60% Histodenz (Sigma-Aldrich) gradient. Parasites were released from iRBCs by saponin lysis, resuspended in Urea extraction buffer (EMD Millipore) and separated by SDS-PAGE. Proteins were detected using rabbit α-PbHP1 (1:2'000) antibodies. *P. knowlesi* A1-H.1 schizonts were enriched on a density cushion of 55% Nycodenz (Axis-Shield) stock solution (27.6% wt/vol Nycodenz in 10 mM Hepes, pH 7.0) in RPMI-1640 medium. RBCs were lysed using 0.15% saponin/PBS and the resultant parasite pellet was diluted 1:100 in Urea extraction buffer (40 mM Tris-HCl, 1 mM EDTA, 8 M Urea, 5% SDS, 1X protease inhibitor cocktail (Sigma-Aldrich), 1% β-mercaptoethanol), mixed with SDS sample buffer and separated by SDS-PAGE alongside a similarly treated uninfected RBC control. PbHP1 was detected with rabbit α-PvHP1 antibodies (1:5’000).

**Chromatin Immunoprecipitation**

For each ChIP reaction, sonicated chromatin containing 500 ng of DNA was incubated in incubation buffer (0.75% SDS, 5% Triton-X100, 750 mM NaCl, 5 mM EDTA, 2.5 mM EGTA, 100 mM Hepes, pH 7.4) with either 1 μg rabbit α-PHP1 (for *P. falciparum*), 1 μg rabbit α-PvHP1 (for *P. vivax* and *P. knowlesi*) or 1 μg rabbit α-PbHP1 (for *P. berghei*, *P. chabaudi* and *P. yoelii*), as well as 10 μl protA and 10 μl protG Dynabeads suspension (Thermo Fisher Scientific).

For each sample four ChIP reactions were prepared and incubated overnight at 4°C while rotating. Beads were washed twice with wash buffer 2 (0.1% SDS, 0.1% DOC, 1% Triton-X100, 150 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 20 mM Hepes, pH 7.4), once with wash buffer 2 (0.1% SDS, 0.1% DOC, 1% Triton-X100, 500 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 20 mM Hepes, pH 7.4), once with wash buffer 3 (250 mM LiCl, 0.5% DOC, 0.5% NP-40, 1 mM EDTA, 0.5 mM EGTA, 20 mM Hepes, pH 7.4) and twice with wash buffer 4 (1 mM EDTA, 0.5 mM EGTA, 20 mM Hepes, pH 7.4). Each wash was performed for 5 min at 4°C while rotating. Subsequently, immunoprecipitated chromatin was eluted in elution buffer (1% SDS, 0.1M NaHCO₃) at room temperature. The eluted chromatin samples and the corresponding input samples (sonicated input chromatin containing 500 ng DNA) were de-crosslinked in 1% SDS/0.1 M NaHCO₃/1 M NaCl at 45°C overnight while shaking. For each parasite strain or species the separate ChIP samples were combined and the DNA was purified using QIAquick MinElute PCR columns (Qiagen).

**High-Throughput Sequencing**

For each sequencing library 2–10 ng of ChIP or input DNA were end-repaired, extended with A-overhangs and ligated to barcoded NextFlex adapters (Bio Scientific) as described previously (Hoeijmakers et al., 2011). Libraries were amplified (98°C for 2 min; four cycles 98°C for 20 sec, 62°C for 3 min; 62°C for 5 min) using KAPA HiFi HotStart ready mix (KAPA Biosystems) and NextFlex primer mix (Bio Scientific) as described (Kenschke et al., 2016). 225-325 bp fragments (including the 125 bp NextFlex adapter) were size-selected using a 2% E-Gel Size Select agarose gel (Thermo Fisher Scientific) and amplified by PCR for eight or ten cycles (Table S7) under the same condition as described above. Library purification and removal of adapter dimers was performed with Agencourt AMPure XP beads in a 1:1 library:beads ratio (Beckman Coulter). ChIP-seq libraries were sequenced for 75 bp single-end reads using the NextSeq 500/550 High Output v2 kit (Illumina) on the Illumina NextSeq 500 system.

**QUANTIFICATION AND STATISTICAL ANALYSIS**

**High-Throughput Sequencing Data Analysis**

Using BWA samse (v0.7.12-r1039) (Li and Durbin, 2009) sequencing reads were mapped to the respective reference genomes available on PlasmoDB v26, namely *P. berghei* ANKA, *P. chabaudi chabaudi*, *P. yoelii yoelii* YM, *P. falciparum* 3D7, *P. knowlesi* H and *P. vivax* P01 (PlasmoDB v29). Reads from the *P. falciparum* PI2004 ChIP-seq libraries were additionally mapped against a PI2004 genome assembly, which was obtained after long-read PacBio sequencing in the framework of the PIK3 reference project.

Mapped reads were filtered to mapping quality ≥ 15 (SAMtools v1.2) (Li et al., 2009) and only uniquely mapped reads (3.4-22 million reads for α-P1 ChIP samples and 6.5-55 million reads for input samples) were used for further analysis (Table S7). ChIP-seq data were visualized in the UCSC Genome browser (https://genome-store.ucsc.edu/). All libraries were normalized to the number of mapped reads per million (RPM) and bedgraph files were generated using BEDTools (v2.20.1) (Quinlan and Hall, 2010). For log2 ratio tracks α-P1 ChIP values were divided by input values and log2-transformed using BEDTools (v2.20.1) (Quinlan and Hall, 2010). Within the UCSC genome browser tracks were smoothened and the windowing function was set as ‘mean’.

To calculate the HP1 coverage for individual genes, tags were counted in a 1000 bp window (ATG ± 500 bp) for each coding sequence and offset by +1 to avoid division by zero while calculating fold changes in coverage. α-P1 ChIP-seq and input tag counts were normalized to the number of reads per kb per million mapped reads (RPKM). ChIP-seq enrichment values were calculated as α-P1 ChIP [RPKM]/input [RPKM] and genes encoded by the mitochondrial or apicoplast genomes and nuclear genes with low mappability (input RPKM < 5) were excluded from downstream analysis.
To visualize the genome-wide HP1 coverage in schizont stages of the six *Plasmodium* species (*P. falciparum*, *P. yoelii*, *P. chabaudi*, *P. knowlesi*, *P. vivax*, *P. falciparum*) the respective reference genomes were divided into 1000 bp windows using BEDTools (v2.20.1) (Quinlan and Hall, 2010). For each window ChIP-seq enrichment values were calculated as described above, log₂-transformed and visualized using the software SignalMap v2.0 (www.sequencing.roche.com). Windows with less than five tag counts in the ChIP-seq and/or the input sample were set to '0' and defined as regions with low mappability. To identify and compare the sets of heterochromatic genes across the six *Plasmodium* species ChIP-seq enrichment values were calculated and assigned to either a ‘heterochromatic’ or ‘euchromatic’ compartment. To do so, we fitted a bivariate Gaussian mixture model to the data and calculated the probabilities (p) for genes to belong to either one of the two compartments using the modelling tool ‘normalmixEM’ from the R package ‘mixtools’. For further analysis genes with p > 0.99999 for the ‘heterochromatic’ compartment were considered high confidence heterochromatic genes. Genes with 0.99999 > p > 0.95 were considered potential heterochromatic genes and genes with p < 0.95 were placed in the ‘euchromatic’ compartment. To look for orthologs and syntenic orthologs, high confidence heterochromatic genes (p > 0.99999) for each species were imputed into PlasmoDB v33. For instance, the orthologs (syntenic/non-syntenic) for *P. falciparum* heterochromatic genes (403 genes) were transformed into orthologs of *P. berghei* ANKA, *P. yoelii yoelii* YM, *P. chabaudi chabaudi*, *P. knowlesi* strain H and *P. vivax* P01 using the function ‘transform by orthology’ in PlasmoDB. Similarly, the orthologs for heterochromatic genes in *P. vivax* (834 genes), *P. knowlesi* (355 genes), *P. berghei* (192 genes), *P. chabaudi* (369 genes) and *P. yoelii* (907 genes) were individually transformed into orthologs of the five other *Plasmodium* species. Ortholog sets (syntenic/non-syntenic) among the species were identified using jvenn (Bardou et al., 2014) and assigned according to the species identifier pf, pv, pk, pb, pc or py (Tables S1 and S2).

To investigate the association between heterochromatic region and distribution of *kir* and SICAvargen genes as well as interstitial telomere repeat sequences (ITs) PKHP1 coverage in *P. knowlesi* schizont stages was depicted as described above. Coding sequences of *kit/kir*-like and SICAvargen genes were depicted according to their genomic coordinates within the *P. knowlesi* H genome (PlasmoDB v26). ITs were identified by searching the *P. knowlesi* genome for occurrences of GGTTTA or GGTTTC repeats on both strands using regular expression. The number of these sequences were counted at every 100 bp window and windows with three or more hits were considered (imperfect repeats with mismatches were not considered). To compare PKHP1 occupancy between two clones of *P. knowlesi* (A1–H.1 and A1–C.1) the ratio between PKHP1 occupancy values (ChIP/Input) were calculated for each gene. Based on visual inspection of the UCSC Genome browser tracks we considered 2.5-fold difference as a marked and likely influential change in PKHP1 occupancy. Genes with low mappability (input RPKM < 5) in at least one of the clones were excluded from downstream analyses.

To allow direct comparison of HP1 gene coverage across different *P. falciparum* strains (3D7, Pf2004, NF135 and NF54) PKHP1 ChIP-seq reads from all four strains were mapped against the *P. falciparum* 3D7 reference genome (PlasmoDB v26). ChIP-seq reads from strain Pf2004 schizonts were additionally mapped against the Pf2004 reference genome (Table S7). Note that although matching reference genomes do exist for NF54 and NF135 (Plas_falc_NF54_v1 and Plas_falc_NF135_5_C10_v1; http://protists.ensembl.org) the respective reference genomes were divided into 1000 bp windows using BEDtools (v2.20.1) (Quinlan and Hall, 2010). For each window ChIP-seq enrichment values were calculated as described above, log₂-transformed, averaged across the strains and visualized using the software SignalMap v2.0 (www.sequencing.roche.com). Coding sequences of the genes in k-means clusters 5 to 11 were depicted according to their location within the *P. falciparum* 3D7 genome (PlasmoDB v26).

For the comparison of intra-erythrocytic stages, ChIP-seq enrichment values were calculated using ChIP RPKM values of ring stages (8-16 hpi), trophozoites (24-32 hpi) or schizonts (40-48 hpi) and normalized to the number of reads per kb per million mapped reads. For each gene the maximum transcript abundance value (RPKM) observed during intra-erythrocytic development was plotted in the scatter plot and var genes as well as genes displaying clonally variant expression (Rovira-Graells et al., 2012) were specifically highlighted.

To assess differences in PHHP1 occupancy between Pf2004 schizonts, stage II/III gametocytes and stage IV/V gametocytes ChIP-seq reads were mapped against the *P. falciparum* 3D7 genome (PlasmoDB v26) and the *P. falciparum* Pf2004 reference genome (Table S7). Genes with low mappability (input RPKM < 5) in at least one of the stages were excluded from downstream analysis. For the remaining genes ChIP-seq enrichment values were z-score transformed, k-means clustered and depicted as a heatmap using the R package ‘pheatmap’. To visualize the genome-wide PHHP1 occupancy for schizont stages and stage IV/V gametocytes (Figure 6C) the *P. falciparum* 3D7 reference genome was divided into 1000 bp windows using BEDTools (v2.20.1) (Quinlan and Hall, 2010). For each window ChIP-seq enrichment values were calculated as described above, z-score transformed and visualized using the software SignalMap v2.0 (www.sequencing.roche.com). Coding sequences of the genes in k-means clusters 5 and 6 to 8
were depicted according to their location within the *P. falciparum* 3D7 genome (PlasmoDB v26). Additionally, we visually compared our Pf2004 PfHP1 ChIP-seq data with the midgut oocyst and salivary gland sporozoite H3K9me3 ChIP-seq datasets generated by Gómez-Díaz and colleagues (Gómez-Díaz et al., 2017) using the UCSC genome browser (https://genome-store.ucsc.edu/). Midgut oocyst and salivary gland sporozoite H3K9me3 ChIP-seq and input data (GEO accession numbers GSM1981878, GSM1981880, GSM1981883, GSM1981885) were aligned to the *P. falciparum* 3D7 reference genome (PlasmoDB v26) (Table S7) and processed as described above to generate bedgraph log2 H3K9me3-ChIP/Input ratio files.

**DATA AND SOFTWARE AVAILABILITY**

The accession number for the ChIP-seq data reported in this paper is GEO: GSE102695. The sequence and annotation of the Pf2004 genome is available at ftp://ftp.sanger.ac.uk/pub/project/pathogens/Plasmodium/falciparum/PF3K/SecondSetReference Genomes/DraftAnnotation/Pf2004/.