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Heterochromatin Protein 1 Secures Survival and Transmission of Malaria Parasites

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SUMMARY

Clonally variant expression of surface antigens allows the malaria parasite *Plasmodium falciparum* to evade immune recognition during blood stage infection and secure malaria transmission. We demonstrate that heterochromatin protein 1 (HP1), an evolutionary conserved regulator of heritable gene silencing, controls expression of numerous *P. falciparum* virulence genes as well as differentiation into the sexual forms that transmit to mosquitoes. Conditional depletion of *P. falciparum* HP1 (PfHP1) prevents mitotic proliferation of blood stage parasites and disrupts mutually exclusive expression and antigenic variation of the major virulence factor PfEMP1. Additionally, PfHP1-dependent regulation of PfAP2-G, a transcription factor required for gametocyte conversion, controls the switch from asexual proliferation to sexual differentiation, providing insight into the epigenetic mechanisms underlying gametocyte commitment. These findings show that PfHP1 is centrally involved in clonally variant gene expression and sexual differentiation *in P. falciparum* and have major implications for developing antidisease and transmission-blocking interventions against malaria.

INTRODUCTION

The protozoan parasite *Plasmodium falciparum* elicits the most severe form of malaria in humans and causes several hundred million clinical cases and 700,000 deaths annually (World Health Organisation, 2013). Malaria morbidity and mortality occur due to the massive expansion of the parasite population during blood-stage infection. Here, parasites mature intracellularly through the ring and trophozoite stages, before successive S/M phases produce a multinucleated schizont that releases up to 32 merozoites ready to invade new red blood cells (RBCs). In order to secure survival and establish chronic blood-stage infection, *P. falciparum* employs clonally variant gene expression (CVGE) as a means to adapt to environmental challenges in the human host, in particular those imposed by the immune system (Rovira-Graells et al., 2012; Cortés et al., 2012).

The most striking example of CVGE is erythrocyte membrane protein 1 (PfEMP1), the major antigen and prime immune target on the surface of infected RBCs (iRBCs) (Scherf et al., 2008). PfEMP1 is encoded by the 60-member *var* gene family (Su et al., 1995; Baruch et al., 1995) and mediates cytoadherence of iRBCs to microvascular endothelium, which prevents parasite clearance in the spleen and causes pathology that contributes substantially to severe malaria outcomes (Kyes et al., 2001). *var* transcription conforms to the concept of singular gene choice (or mutual exclusion); in each parasite only a single *var* gene is active, while all other members remain silenced (Scherf et al., 1998). Transcriptional switches in *var* gene expression result in CVGE and consequently antigenic variation of PfEMP1 and immune evasion (Smith et al., 1995; Scherf et al., 1998). Importantly, this survival strategy is directly linked to malaria transmission; during each replicative cycle a small number of parasites commit to sexual development and differentiate into mature stage V gametocytes, the only stage capable of transmitting the infection to the mosquito vector (Baker, 2010).

Singular *var* gene choice is regulated by a poorly understood interplay between transcriptional and epigenetic control mechanisms (Guizetti and Scherf, 2013). Particularly striking is the observation that *var* genes are associated with histone 3 lysine 9 trimethylation (H3K9me3) and heterochromatin protein 1 (HP1) (Salcedo-Amaya et al., 2009; Lopez-Rubio et al., 2009; Flueck et al., 2009; Pérez-Toledo et al., 2009; Chookajorn et al., 2007; Lopez-Rubio et al., 2007). HP1 is an evolutionarily conserved regulator of heterochromatin formation and heritable gene silencing and was originally described in *Drosophila melanogaster* as a suppressor of position effect variegation (Eissenberg et al., 1990). HP1 binds to H3K9me2/H3K9me3, the hallmark histone modification of heterochromatin, and recruits H3K9-specific methyltransferases (HKMTs) that modify adjacent nucleosomes (Lomberk et al., 2006). As a result, HP1 sustains a self-perpetuating mechanism for heterochromatin spreading and heritable gene silencing. In addition, HP1 also regulates euchromatic genes and is involved in other chromatin-related processes, including cohesion, telomere maintenance, or DNA replication and repair (Kwon and Workman, 2008). This functional versatility is linked to the evolution of HP1 paralogs, particularly in metazoans, and the ability of HP1 to recruit functionally

diverse proteins (Lomberk et al., 2006; Kwon and Workman, 2008).

P. falciparum contains only a single HP1 protein that localizes primarily to H3K9me3-enriched heterochromatic regions. These chromosomal domains incorporate all *var* genes and hundreds of other clonally variant genes (such as *rif*, *stevor*, and *pfmc-2tm*) encoding species-specific blood-stage antigens. At the same time, PfHP1 is also found at a small number of euchromatic loci (Flueck et al., 2009; Pérez-Toledo et al., 2009). PfHP1 overexpression leads to increased silencing of some heterochromatic genes (Flueck et al., 2009), and the presence or absence of PfHP1 is linked to the silenced or active state of *var* genes, respectively (Pérez-Toledo et al., 2009). Together, these observations suggest key functions for PfHP1 in heritable silencing and phenotypic variation of a large set of factors implicated in host-parasite interactions and immune evasion. However, if and to what extent PfHP1 is indeed required for mutually exclusive *var* expression and/or for CVGE in general is unknown. Moreover, since the *pfhp1* locus is refractory to genetic deletion (Flueck et al., 2009; Pérez-Toledo et al., 2009), additional unknown HP1-dependent pathways essential for parasite proliferation are likely to exist in *P. falciparum*.

Here, we conducted a comprehensive functional analysis of PfHP1 by generating a conditional PfHP1 loss-of-function mutant. We show that PfHP1 is indispensable for the heritable silencing of heterochromatic genes in general and in particular for the maintenance of singular *var* gene choice and antigenic variation of PfEMP1. In addition, PfHP1 is required at the G1/S transition phase for mitotic proliferation of blood-stage parasites. Intriguingly, we also discovered that PfHP1 controls sexual commitment by regulating the bistable expression of single euchromatic locus encoding an ApiAP2 transcription factor.

RESULTS

PfHP1 Is Indispensable for Mitotic Proliferation of Blood-Stage Parasites

We applied the FKBP destabilization domain (DD) technique that allows modulating expression levels through the stabilizing compound Shield-1 (Banaszynski et al., 2006; Armstrong and Goldberg, 2007) and generated a clonal parasite line expressing endogenous PfHP1 as a C-terminally tagged GFP-DD fusion (3D7/HP1^{ON}) (Figure S1, available online). In the presence of Shield-1, 3D7/HP1^{ON} parasites exhibited no growth phenotype (Figure 1A) and multiplied at a rate within a single asexual replication cycle (3.8-fold ± 0.6 SD) similar to that of 3D7/HP1^{ctrl} parasites in which PfHP1 is tagged with GFP only (4.4-fold ± 0.4 SD). When Shield-1 was withdrawn at 4–12 hr postinvasion (hpi), 3D7/HP1^{OFF} parasites completed the current intraerythrocytic developmental cycle (IDC) and subsequent ring-stage development with normal kinetics. Strikingly, however, these parasites arrested prior to schizogony in generation 2 (Figure 1A), and all efforts to select for proliferating subpopulations were unsuccessful.

Live-cell imaging revealed the expected perinuclear localization of tagged PfHP1 in 3D7/HP1^{ON} and 3D7/HP1^{ctrl} parasites throughout the IDC, whereas in 3D7/HP1^{OFF} parasites PfHP1 was undetectable 12 hr after Shield-1 withdrawal (Figure 1B). A more direct assessment by parallel western blot and immunofluorescence assays (IFA) showed that after Shield-1 removal at 4–12 hpi, PfHP1 was still detectable but reduced in late ring stages (16–24 hpi) and early schizonts (32–40 hpi) and localized diffusely to the nucleoplasm and cytoplasm (Figure 1C). After reinvasion, PfHP1 was undetectable in 3D7/HP1^{OFF} parasites by both methods. Similarly, targeted chromatin immunoprecipitation (ChIP-qPCR) showed that PfHP1 occupancy at subtelomeric (PF3D7_0426000) and chromosome-internal (PF3D7_0412400) *var* loci was unchanged in late ring stages but substantially reduced in schizonts and subsequent generation 2 ring stages (Figure 1D).

We next analyzed parasite viability using isothermal microcalorimetry (Wenzler et al., 2012). In generation 1, 3D7/HP1^{ON} and 3D7/HP1^{OFF} populations both displayed a typical heat emission profile marked by increased heat flow in trophozoites and schizonts (Figure 1E). In generation 2, however, the metabolic activity in PfHP1-depleted parasites changed dramatically, and heat emission remained low over the entire 48 hr period of measurement. Importantly, these parasites were still viable since they emitted heat at a rate significantly higher than that of uninfected RBCs.

PfHP1 Controls Sexual Differentiation

Intriguingly, prolonged microscopic observation revealed that PfHP1-depleted parasites consisted of a mixture of growth-arrested trophozoites and sexual forms undergoing gametocyte development (Figure 2A). Note that sexual conversion occurs through an unknown mechanism during the cell cycle prior to gametocyte development and that all daughter parasites released from a committed schizont undergo sexual differentiation (Bruce et al., 1990). To discriminate quantitatively between growth-arrested and sexual forms, we visualized the gametocyte-specific marker Pfs16 (Bruce et al., 1994) and knob-associated histidine-rich protein (KAHRP) (a marker for iRBCs) (Taylor et al., 1987) by indirect IFA. Remarkably, 52.7% (±3.1 SD) of 3D7/HP1^{OFF} parasites expressed Pfs16 in generation 2, compared to only 2.3% (±1.2 SD) of background conversion in the 3D7/HP1^{ON} population (Figure 2B). Overview images of a Giemsa-stained blood smear (6 days postinvasion) and an α-Pfs16 IFA experiment (32–40 hpi) provide visual confirmation of this phenotype showing a high proportion of stage II/III and stage I gametocytes, respectively, in 3D7/HP1^{OFF} parasites (Figures 2C and 2D). Notably, PfHP1-depleted gametocytes completed sexual development within 8–10 days, similar to control gametocytes (Figure S2). Hence, PfHP1 depletion triggers the synchronous hyperinduction of viable gametocytes, which demonstrates that sexual commitment in malaria parasites is epigenetically regulated.

PfHP1-Depleted Asexual Parasites Enter a Reversible Cell-Cycle Arrest

To investigate at which stage of the cell cycle the nongametocyte subpopulation of 3D7/HP1^{OFF} parasites arrested, we performed single-cell DNA content analysis by flow cytometry. This revealed that in contrast to 3D7/HP1^{ON} parasites, virtually all parasites in the 3D7/HP1^{OFF} population failed to replicate their genome in generation 2 (Figure 3A). While this is expected for nonproliferative gametocytes, this result demonstrates that the population of asexual parasites arrested prior to or during the

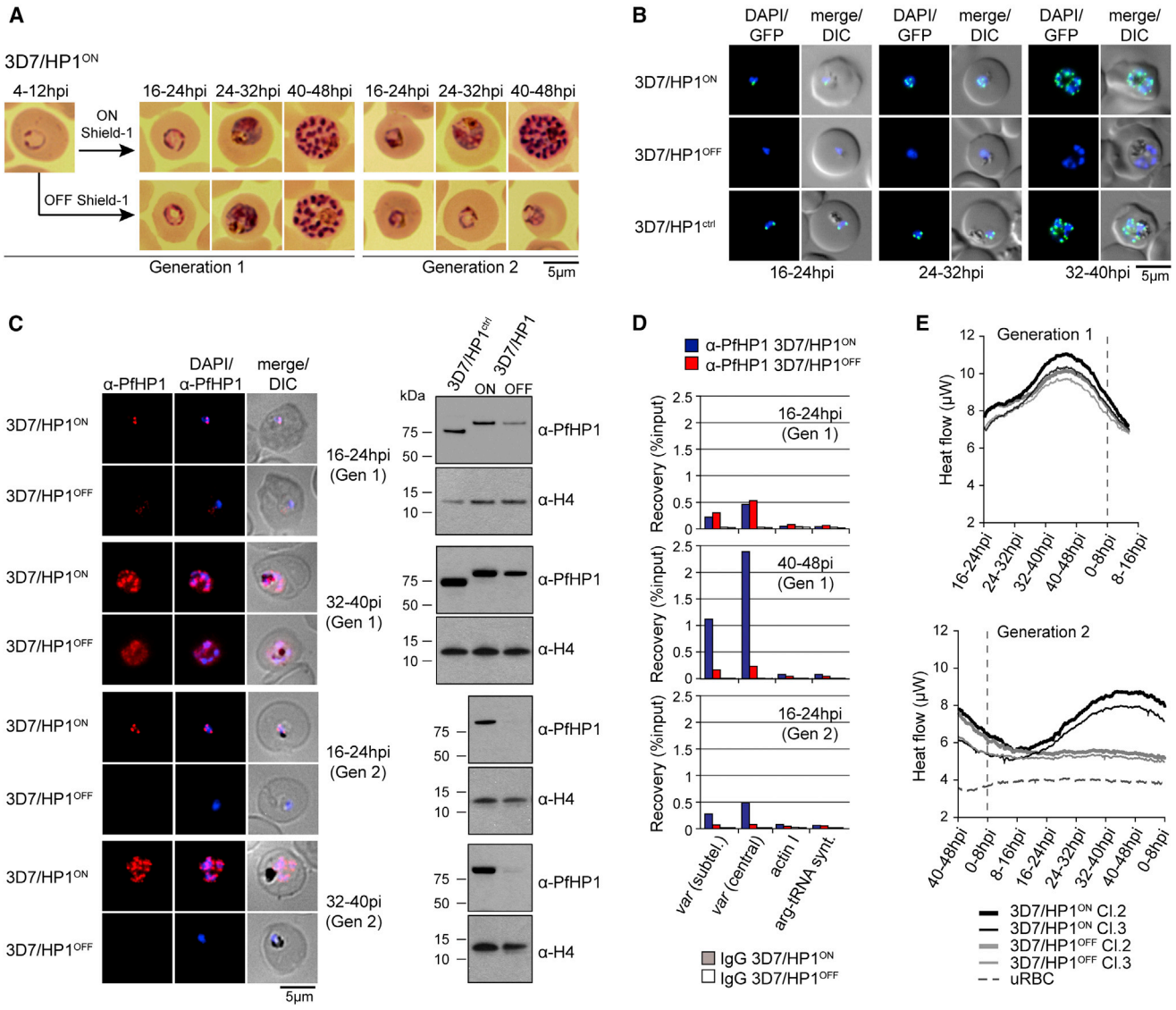

Figure 1. Growth Phenotype of a Conditional PfHP1 Loss-of-Function Mutant and Kinetics of PfHP1 Depletion

(A) Giemsa-stained blood smears showing development of 3D7/HP1^{ON} and 3D7/HP1^{OFF} parasites over two generations (96 hr). See also Figure S1.

(B) Expression and localization of PfHP1 in 3D7/HP1^{ON}, 3D7/HP1^{OFF}, and 3D7/HP1^{ctrl} parasites by live fluorescence microscopy (images taken 12 hr after removal of Shield-1).

(C) Expression and localization of PfHP1 in 3D7/HP1^{ON} and 3D7/HP1^{OFF} parasites by IFA and western blot (Shield-1 removal at 4–12 hpi). The production and specificity of affinity-purified polyclonal α-PfHP1 antibodies is described in Figure S1 and the Supplemental Experimental Procedures.

(D) PfHP1 occupancy at two heterochromatic *var* and two euchromatic control loci in 3D7/HP1^{ON} and 3D7/HP1^{OFF} parasites was determined by ChIP-qPCR (Shield-1 removal at 4–12 hpi). See also Figure S6.

(E) Heat emission as determined by isothermal microcalorimetry in two 3D7/HP1 clones (Cl.2 and Cl.3) grown in the presence or absence of Shield-1. uRBC, uninfected RBCs.

first S phase of schizogony. Interestingly, this cell-cycle defect was reversible since PfHP1-depleted trophozoites reentered S phase and mitotic proliferation when Shield-1 was added back to the culture medium (Figure 3B). Even after 12 days in the absence of Shield-1, rescued trophozoites reaccumulated perinuclear PfHP1 and progressed through schizogony (Figure 3C). This was not due to a genetic reversion, as rescued parasites entered developmental arrest and gametocyte hyperconversion when Shield-1 was withdrawn for a second time (data not shown). With prolonged time in the absence of Shield-1, however, the parasitemia decreased, and the time required for growth resumption after Shield-1 replenishment increased, showing that a subset of PfHP1-depleted parasites died over time (Figures 3B and 3D). Together, these data corroborate the essential function of PfHP1 in mitotic proliferation and show that a subset of PfHP1-depleted trophozoites remained in a state of dormancy capable of reentering the cell cycle if PfHP1 expression was restored.

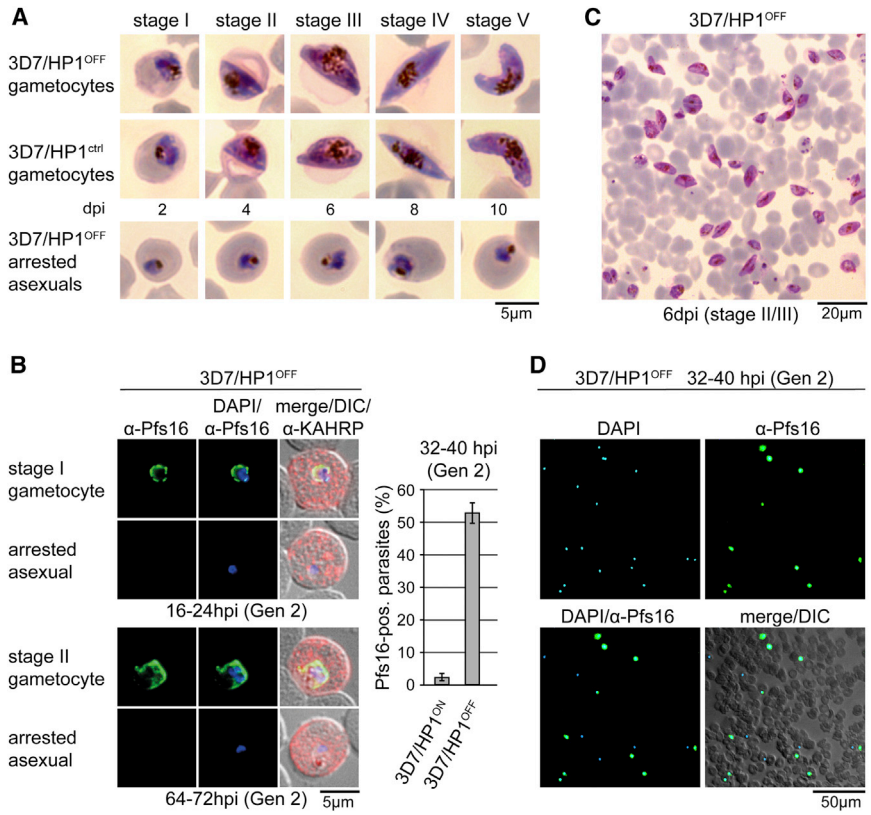

Figure 2. PfHP1 Depletion Induces Gametocyte Conversion

(A) 3D7/HP1^{OFF} and 3D7/HP1^{ctrl} gametocytes and cell-cycle-arrested 3D7/HP1^{OFF} trophozoites. dpi, days postreinvasion.

(B) Distinction between 3D7/HP1^{OFF} early gametocytes and arrested trophozoites by IFA (left) and proportion of Pfs16/KAHRP-positive parasites in 3D7/HP1^{ON} and 3D7/HP1^{OFF} (right). Values show the mean ± SD of three biological replicates (100 KAHRP-pos. iRBCs were scored per experiment).

(C) Giemsa-stained blood smear of a 3D7/HP1^{OFF} parasite culture (Shield-1 removal at 4–12 hpi) at 6 days postreinvasion (dpi) (image taken at 60× magnification). The gametocyte hyperinduction phenotype is highlighted by the high proportion of stage II/III gametocytes among all iRBCs.

(D) α-Pfs16 IFA of a 3D7/HP1^{OFF} parasite culture (Shield-1 removal at 4–12 hpi) at 32–40 hr postreinvasion (image taken at 40× magnification). The gametocyte hyperinduction phenotype is highlighted by the high proportion of Pfs16-positive stage I gametocytes among all DAPI-positive iRBCs. See also Figure S2.

Lack of S/M Phase Entry Correlates with Decelerated Transcriptome Progression in G1 Phase

We next conducted genome-wide transcriptional profiling of paired synchronous 3D7/HP1^{ON} and 3D7/HP1^{OFF} cultures at 11 consecutive time points (TPs) spanning generations 1 and 2 to (i) study the effect of PfHP1 on heritable gene silencing and (ii) identify the PfHP1-dependent pathway responsible for gametocyte conversion (Figure 4A and Table S1). Until 16–24 hpi in generation 2, the corresponding transcriptomes were highly comparable between both populations (Figure 4A) and progressed with similar kinetics through the first IDC and second-generation ring-stage development (TPs 2–9) (Figures 4B and 4C). In contrast, at 24–48 hr after reinvasion (TPs 10–12), when 3D7/HP1^{ON} parasites went through schizogony and the 3D7/HP1^{OFF} population consisted of a mixture of early gametocytes and arrested trophozoites, the transcriptomes correlated poorly (Figure 4A), and parasites failed to launch a schizont-specific transcription profile (Figures 4B and 4C). This slowdown in transcriptome development reflects a substantial deceleration in G1 progression and failure to enter S phase in generation 2, which is consistent with the growth phenotype observed for 3D7/HP1^{OFF} parasites.

PfHP1 Silences Heterochromatic Genes and Is Essential for the Maintenance of Singular *Var* Gene Choice

To identify genes differentially expressed in direct response to PfHP1 depletion, we focused our analysis on the comparable growth phase ranging from generation 1 trophozoites to late ring stages in generation 2 (TPs 4–9). Consistent with the conserved role for HP1 in heritable gene silencing, we observed a general derepression of heterochromatic genes in 3D7/HP1^{OFF} parasites, and 113 PfHP1-associated genes (31.2%) displayed a significant increase in mean expression (>1.5-fold, false discovery rate [FDR] < 0.1) compared to 3D7/HP1^{ON} parasites (Figure 5A and Table S1). In contrast, only 16 euchromatic genes (0.34%) were differentially expressed, of which four upregulated genes had previously been associated with early gametocyte development: PF3D7_1102500 (*phistb*; GEXP02), PF3D7_1335000 (*msrp1*), PF3D7_1472200 (class II histone deacetylase [HDAC]), and PF3D7_1473700 (*nup116*) (Silvestrini et al., 2010; Eksi et al., 2012) (Figure 5B and Table S1). Strikingly, the strongest derepression was observed for the *var* gene family; 52 out of 60 members were significantly and highly upregulated in PfHP1-depleted parasites. In addition, many *rif* and *pfmc-2tm* genes and several members of other subtelomeric gene families were significantly induced, and even among the nonsignificantly deregulated heterochromatic genes, the majority was still upregulated in the absence of PfHP1 (Figures 5A and S3).

We next investigated the prevailing role of PfHP1 in *var* gene silencing in more detail. Removal of Shield-1 at 4–12 hpi had no immediate effect on *var* transcription in generation 1 (Figure 6A), which is explained by the persistent binding of PfHP1 to chromatin shortly after Shield-1 withdrawal (Figure 1D). *var* transcription was also unchanged in schizonts, demonstrating that *var* promoters retain their ring-stage-specific activation profile even in the absence of PfHP1. By contrast, almost all *var* genes were massively upregulated after reinvasion, and individual genes showed expression levels up to 30-fold higher (Figure 6A). Importantly, however, the few *var* genes already dominantly expressed in 3D7/HP1^{ON} parasites, most notably *var2csa* (PF3D7_1200600) (Salanti et al., 2003), were not or

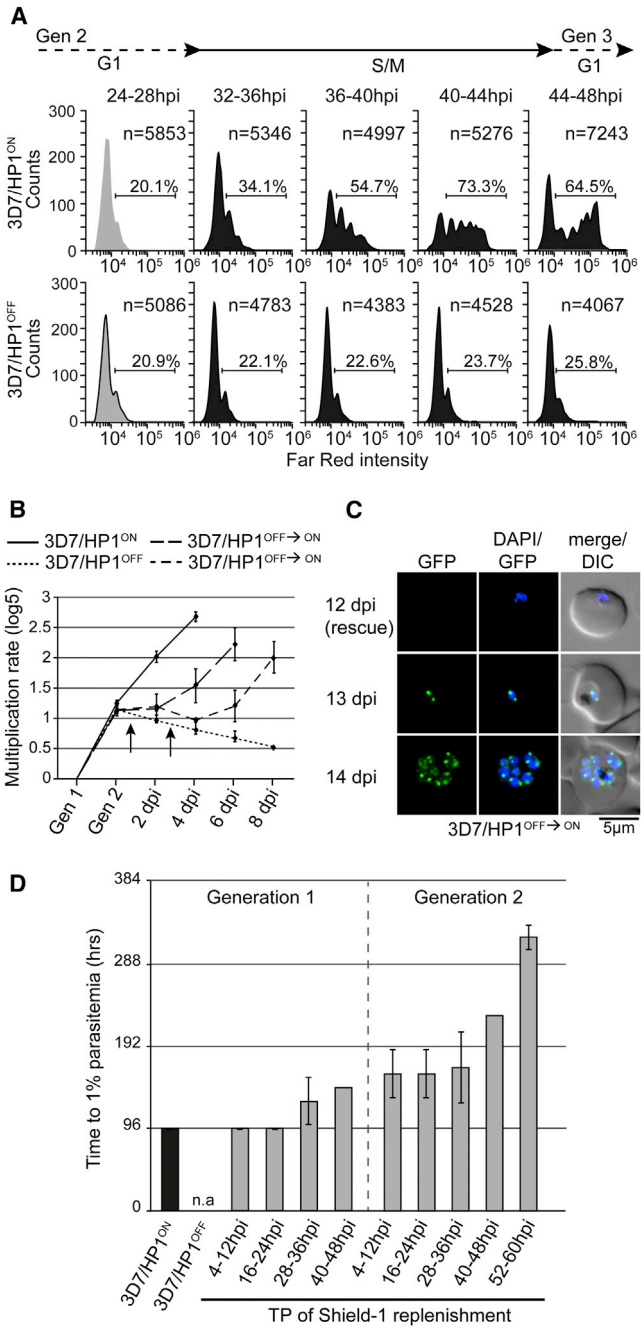

Figure 3. PfHP1 Depletion Causes Reversible Cell-Cycle Arrest at the G1/S Transition Phase

(A) Flow cytometry analysis of genomic DNA content in 3D7/HP1^{ON} and 3D7/HP1^{OFF} parasites at five consecutive time points in generation 2. The percentage of iRBCs with ≥2 genomes is indicated. Prior to S phase (gray), this value corresponds to RBCs infected with ≥2 parasites (confirmed by microscopy). *n*, number of gated iRBCs.

(B) Cell-cycle-arrested 3D7/HP1^{OFF} parasites reestablish asexual growth after adding back Shield-1 at 24 or 72 hr postreinvasion (arrows). Values show the mean of three biological replicates ± SD.

(C) Growth-arrested 3D7/HP1^{OFF} parasites reenter mitotic proliferation after Shield-1 replenishment. dpi, days postreinvasion.

(D) Synchronous 3D7/HP1^{ON} cultures (~0.1% parasitemia) were split at 0–8 hpi and cultured in either the presence or absence of Shield-1. Shield-1 was added back to 3D7/HP1^{OFF} cultures at nine consecutive TPs. Cultures were smeared daily and analyzed by Giemsa staining until they reached a parasitemia of >1%. Values show the mean of three biological replicates ± SD.

only slightly induced (Figures 6A and S4). This proves that *var* activation was not due to transcriptional switches but that all *var* genes were active simultaneously in 3D7/HP1^{OFF} parasites. Consistent with these findings, PfHP1-depleted parasites coexpressed several PfEMP1 variants of different sizes, whereas 3D7/HP1^{ON} parasites predominantly expressed a single protein consistent with the size of VAR2CSA (Figure 6B). IFAs further corroborated hyperexpression of PfEMP1 in 3D7/HP1^{OFF} parasites at the single-cell level and indicated correct trafficking of PfEMP1 to the iRBC surface (Figure 6C).

Sexual Differentiation Is Linked to the PfHP1-Dependent Derepression of the ApiAP2 Transcription Factor PfAP2-G

Interestingly, a single member of the *apiap2* family of genes encoding phylum-specific transcription factors (TFs) (Balaji et al., 2005) was also significantly derepressed in 3D7/HP1^{OFF} parasites (Figure 5A). This *apiap2* gene (PF3D7_1222600) represents the only PfHP1-associated member of the family (Flueck et al., 2009) and encodes the TF AP2-G that is essential for gametocyte conversion in *P. falciparum* and *P. berghei* (Kafsack et al., 2014; Sinha et al., 2014). Moreover, among all deregulated PfHP1-associated loci, *pfap2-g* was the only gene that does not encode a surface antigen or exported protein (Table S1). We observed that *pfap2-g* derepression was already initiated in 3D7/HP1^{OFF} generation 1 schizonts (32–40 hpi), coincident with the dissociation of PfHP1 from the *pfap2-g* locus (Figure 7A). Importantly, when 3D7/HP1^{OFF} parasites were allowed to reaccumulate PfHP1 prior to schizogony (28–36 hpi), gametocyte hyperconversion was prevented (Figures 7B and S5). Restoring PfHP1 expression at 34–42 hpi was only moderately effective in preventing sexual commitment, and parasites rescued at 40–48 hpi or after reinvasion showed a hyperconversion phenotype similar to that of nonrescued parasites. The temporal correlation between derepression of *pfap2-g* and gametocyte commitment during schizogony, together with the fact that both processes are strictly PfHP1 dependent, identifies the targeted activation of PfAP2-G as the key mechanism responsible for sexual conversion.

PfHP1 Depletion Results in Reduced H3K9me3 Levels at Heterochromatic Loci

HP1-dependent recruitment of SU(VAR)3-9-type HKMTs is essential for the spreading and inheritance of H3K9me3 marks in model eukaryotes (Grewal and Jia, 2007). We therefore tested if the local depletion of PfHP1 caused a reduction of H3K9me3 levels. Indeed, ChIP-qPCR experiments demonstrated that H3K9me3 occupancy was substantially reduced at *var* genes and the *pfap2-g* locus in 3D7/HP1^{OFF} parasites, in both generation 1 schizonts and generation 2 ring stages (Figure S6). Notably, the drop in H3K9me3 enrichment at individual loci was pronounced to a degree equal to that of the depletion of PfHP1 itself. As expected, PfHP1 and H3K9me3 were not associated with the early gametocyte marker *pfs16* in both cultures at both TPs, which confirms that upregulation of *pfs16* in early gametocytes is PfHP1 independent and rather occurs as a result of

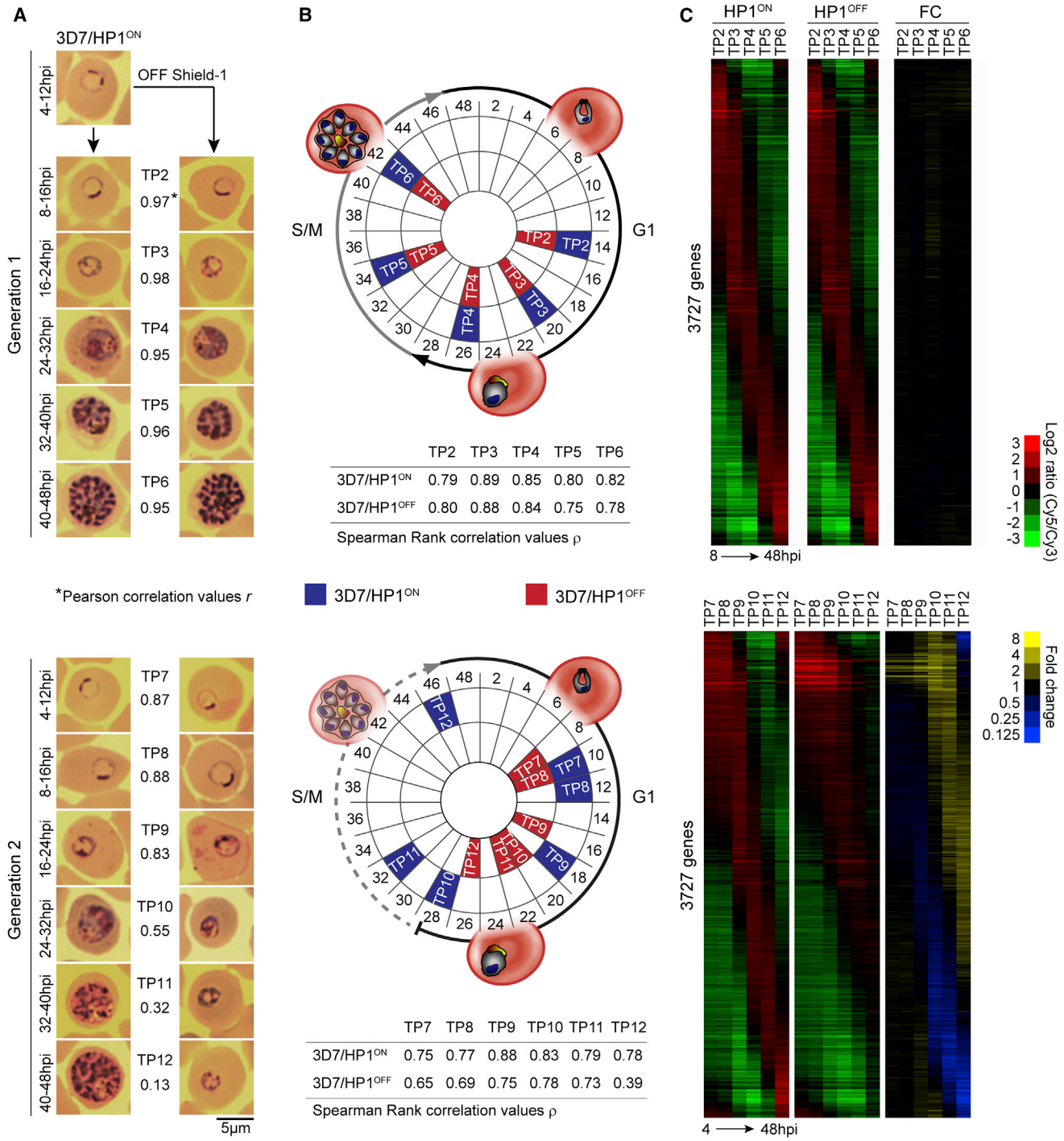

Figure 4. *Pf*HP1 Depletion Leads to a Marked Slowdown in Transcriptome Progression

(A) 3D7/HP1^{ON} and 3D7/HP1^{OFF} parasites were sampled for comparative transcriptome analysis. Pairwise correlation between transcriptomes of corresponding TPs is indicated by Pearson correlation coefficient *r* (asterisk).

(B) Mapping of experimental transcriptomes to a high-resolution reference data set (Mok et al., 2011). Blue and red boxes identify the best-fit TP (hpi) in a high-resolution reference data set for each 3D7/HP1^{ON} and 3D7/HP1^{OFF} transcriptome, respectively. Spearman rank coefficients (ρ) are provided. See also Supplemental Experimental Procedures.

(C) Comparison of global temporal expression profiles in generations 1 and 2. Heatmaps are ordered according to the phase calculated for 3D7/HP1^{ON} parasites (TPs 7–12, starting at $-\pi/2$) and display relative gene expression levels (red/green) and fold changes (FCs) in gene expression (yellow/blue). See also Table S1 and Supplemental Experimental Procedures.

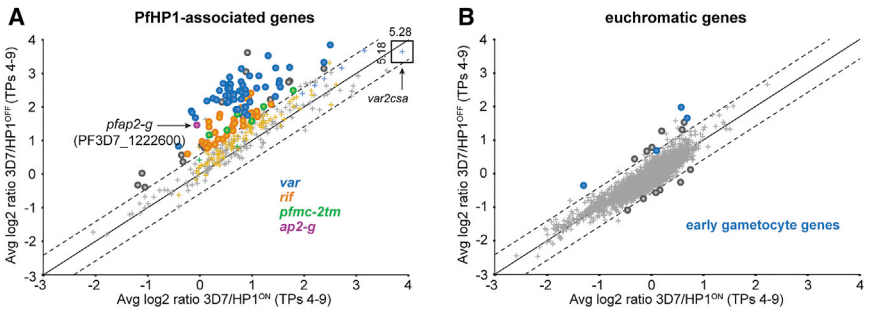

Figure 5. PfHP1 Depletion Leads to Derepression of PfHP1-Associated Genes

(A) Scatter plot comparing mean relative expression values of all 362 PfHP1-associated genes. Significantly deregulated genes are indicated by circles (>1.5-fold; FDR < 0.1). See also Figure S3 and Table S1.

(B) Scatter plot comparing mean relative expression values of all 4,771 euchromatic genes. Significantly deregulated genes are indicated by circles (>1.5-fold; FDR < 0.1). See also Table S1.

a gametocyte-specific transcriptional program. Together, these findings show that, in analogy to other eukaryotes, PfHP1 is required for the local deposition and inheritance of H3K9me3 marks on newly replicated chromatin. This is likely mediated by the PfHP1-dependent recruitment of a H3K9-specific HKMT (probably PfSET3; Volz et al., 2010; Lopez-Rubio et al., 2009). However, confirmation of PfSET3 as a functional SU(VAR)3-9 homolog as well as a possible physical interaction of this factor with PfHP1 remains to be determined.

Identification of Genes Associated With Early Gametocyte Development

Our experimental setup combined with the high rate of synchronous gametocyte induction in PfHP1-depleted parasites allowed us to identify transcriptional events linked to gametocyte conversion in real time and based on comparison of two isogenic clones. Following derepression of *pfap2-g* in generation 1, known markers of early sexual development were upregulated only after reinvasion (Table S1), and this was confirmed by quantitative RT-PCR (qRT-PCR) (Figure 7C). We therefore queried our data set to identify genes induced upon gametocyte conversion and identified 29 additional early gametocyte candidate genes (Figure 7D and Table S1). Notably, 17 (58.6%) of these genes have been linked to early sexual development in previous high-throughput studies (Silvestrini et al., 2005, 2010; Eksi et al., 2012; Young et al., 2005), which underpins the high accuracy and stringency of our search. qRT-PCR confirmed upregulation of these genes in 3D7/HP1^{OFF} parasites and showed that apart from *pfap2-g*, only one additional gene (PF3D7_0832300; *phista*-like) was upregulated already during the commitment phase. In contrast, induction of all other genes was delayed until the sexual ring stage and increased further during stage I gametocyte development (24–40 hpi) (Figures 7D and S7). Finally, we tested if these candidate genes are also upregulated in naturally induced gametocytes by comparing their transcription between 3D7 wild-type parasites and a gametocyte-deficient clone of 3D7 (F12) (Alano et al., 1995). Indeed, all predicted genes showed consistently higher transcription levels in 3D7 compared to F12, which ultimately confirms that their activation is related to early gametocyte differentiation (Figure S7).

DISCUSSION

Our study shows that PfHP1 is strictly required to propagate nonpermissive heterochromatin to daughter cells in order to silence a vast antigenic repertoire and, in particular, to perpetuate mutually exclusive *var* transcription. Since the landmark discovery of the *var* gene family (Su et al., 1995; Baruch et al., 1995; Smith et al., 1995), a large number of studies firmly established that antigenic variation in *P. falciparum* is controlled by a complex epigenetic strategy involving reversible histone modifications, chromatin remodeling, and locus repositioning (Lopez-Rubio et al., 2007, 2009; Jiang et al., 2013; Freitas-Junior et al., 2005; Tonkin et al., 2009; Duraisingh et al., 2005; Petter et al., 2011; Voss et al., 2006; Ralph et al., 2005). Together, these findings support a model in which singular *var* gene choice is achieved by restricting transcription of a single locus to an elusive perinuclear *var* expression site (VES) and where switching occurs through competitive replacement of the active gene with a previously silenced member. How these different processes and layers of regulation are interconnected to control antigenic variation, however, is only poorly understood.

Here, we demonstrate that depletion of PfHP1 during schizogony leads to the simultaneous activation of all *var* genes and concomitant hyperexpression of PfEMP1 in daughter parasites. This shows that PfHP1 is required to protect *var* genes from activation outside the VES, which is further supported by the fact that *var* promoter fragments activate stage-specific transcription by default when placed upstream of the transcriptional start site of a euchromatic gene (Brancucci et al., 2012). Hence, unlike in African trypanosomes (Navarro and Gull, 2001), the functional principle of the VES is not based on the sequestration of exclusive transcription machinery but rather depends on histone modifying and remodeling activities capable of disassembling heterochromatin at a single locus. This concept is consistent with the recent description of the H3K4me-specific methyltransferase (HKMT) PfSET10 that localizes exclusively to the VES (Volz et al., 2012). Note that mutually exclusive *var* transcription is also disrupted in parasites lacking expression of the H3K36-specific HKMT PfSET2 (also known as PfSETvs) (Jiang et al., 2013). Interestingly, Jiang et al. (2013) observed a reduction in H3K36me3 as well as H3K9me3 occupancy at active *var* loci in ΔPfSET2 parasites, suggesting functional interdependence of different epigenetic control processes in regulating antigenic variation. Taken together, these results fill an important gap in our understanding of the regulatory mechanisms underlying mutually exclusive *var* gene transcription and antigenic variation of PfEMP1 and will be instrumental for the further functional dissection of this important immune evasion strategy. Moreover, the PfEMP1 hyperexpression phenotype reported here will serve as a useful tool to study PfEMP1-based pathogenesis and immunity and may provide opportunities for the development of a

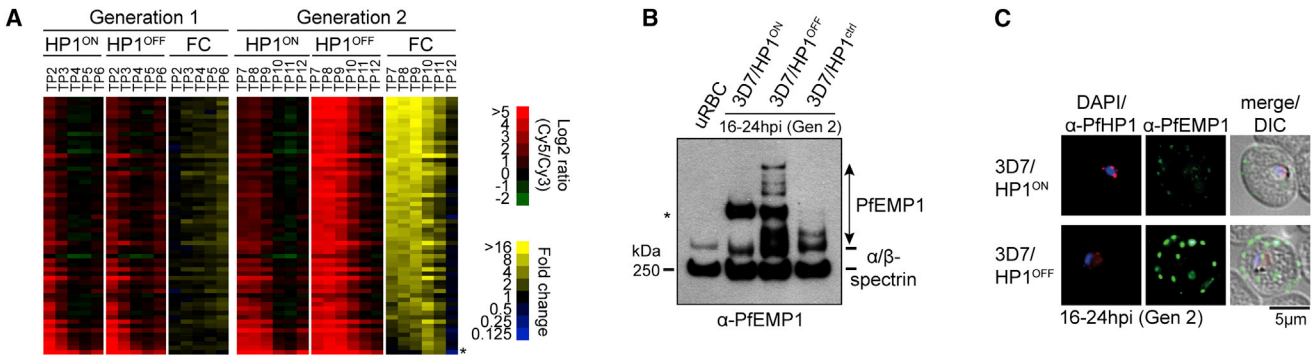

Figure 6. PfHP1 Is Required for Heritable *var* Gene Silencing and Maintenance of Singular *var* Gene Choice

(A) Temporal progression of relative abundance (red/green) and fold change (FC) in expression (yellow/blue) for all *var* genes in 3D7/HP1^{ON} and 3D7/HP1^{OFF} parasites across all 11 TPs analyzed. Asterisk, *var2csa*. See also Figure S4 and Table S1.

(B) PfEMP1 expression in 3D7/HP1^{ON}, 3D7/HP1^{OFF}, and 3D7/HP1^{ctrl} parasites at 16–24 hpi in generation 2. Equal cell numbers were analyzed in each lane. The pan-specific α-PfEMP1 antibody (mAb 6H1) was raised against a part of the C-terminal acidic terminal segment (ATS) domain that is conserved among PfEMP1 variants (Duffy et al., 2002). uRBC, uninfected RBCs (note that α-PfEMP1 antibodies cross-react with human spectrin).

(C) α-PfHP1/α-PfEMP1 (mAb 6H1) IFAs of 3D7/HP1^{ON} and 3D7/HP1^{OFF} parasites at 16–24 hpi in generation 2.

malaria vaccine. Notably, in an analogous system, immunization with mutant *Giardia lamblia* parasites coexpressing many variant-specific surface proteins has successfully been applied to induce strain-transcendent protective immunity in an experimental infection model (Rivero et al., 2010).

In addition to virulence gene silencing, we also identified an essential role for PfHP1 in mitotic proliferation. In the absence of PfHP1, asexual trophozoites fail to proliferate and enter a state of cell-cycle arrest that is reversible in a PfHP1-dependent manner. Although the exact pathway in which PfHP1 is required for cell-cycle progression remains unknown, the lack of significant levels of DNA synthesis in PfHP1-depleted trophozoites is indicative of defects in S phase entry or progression. Indeed, HP1 directly interacts with several factors involved in prereplicative complex assembly and replication initiation or elongation (e.g., CDC18/CDC6, ORCs, MCMs, CAF1) in model eukaryotes (Kwon and Workman, 2008; Christensen and Tye, 2003; Li et al., 2011). Moreover, loss of HP1 function causes delayed replication timing and/or S phase progression defects in *S. pombe*, *D. melanogaster*, and mouse cells (Hayashi et al., 2009; Schwaiger et al., 2010; Quivy et al., 2008). Hence, it is conceivable that PfHP1 may be essential for DNA replication in *P. falciparum*, and further experiments are now required to test this intriguing hypothesis in more detail.

Remarkably, we demonstrate that PfHP1's capacity to regulate the bistable transcription of a single euchromatic gene balances mitotic proliferation and sexual differentiation of malaria blood-stage parasites. In this context, it is notable that silencing of heterochromatic genes in *P. falciparum* is functionally dependent on the sirtuin HDACs PfSIR2A/PfSIR2B (Tonkin et al., 2009; Duraisingh et al., 2005), PfSET2 (Jiang et al., 2013), and the class II HDAC PfHDA2 (Coleman et al., 2014). Of these histone-modifying enzymes, however, only PfHDA2 also controls *pfap2-g* expression, suggesting that PfHP1 and PfHDA2 cooperate in a distinct silencing pathway to also regulate euchromatic genes. It will therefore be interesting to test if PfHP1 and PfHDA2 occur together in a specific silencing complex. Indeed, several class II HDACs interact directly with HP1 and are important for HP1-dependent gene silencing in model eukaryotes (Yamada et al., 2005; Zhang et al., 2002). We propose that epigenetic silencing of *pfap2-g* promotes continuous mitotic proliferation and antagonizes sexual conversion, while local dissociation of PfHP1 from the *pfap2-g* locus activates PfAP2-G expression and triggers sexual conversion and gametocyte differentiation. In analogy to the essential role of ApiAP2 TFs in stage-specific gene expression and parasite development in other life cycle stages (Yuda et al., 2009, 2010; Iwanaga et al., 2012), PfAP2-G likely regulates a transcriptional response effecting gametocyte development and cell-cycle exit. In both *P. falciparum* and *P. berghei*, PfAP2-G binding motifs were indeed found enriched in the upstream region of genes associated with sexual differentiation, and the occurrence of the respective target sites upstream of *pfap2-g* itself further indicates that PfAP2-G may establish an autoregulatory feedback loop (Kafsack et al., 2014; Sinha et al., 2014). Interestingly, we demonstrate that transcriptional changes associated with the early phase of differentiation are limited to a small number of genes but become more pronounced once gametocytes enter stage I development. We explain this by the fact that both asexual and sexually committed schizonts need to produce invasive merozoites capable of establishing RBC infection. In fact, many of the early gametocyte genes predicted here and elsewhere (Eksi et al., 2005; Silvestrini et al., 2010) code for proteins implicated in host cell remodeling, which is indicative for the requirement of gametocyte-specific host cell modifications. While it is possible that PfAP2-G regulates some or all of these genes directly, genome-wide ChIP approaches will be necessary for a comprehensive identification of PfAP2-G target genes and understanding of PfAP2-G function.

Our results reveal important mechanistic insight into the pathway underlying sexual commitment and identify PfHP1 as a crucial factor in controlling cell-fate decision in *P. falciparum*. Interestingly, the blood stage of infection is the only phase of the entire life cycle where parasites have a choice to enter either one of two developmental pathways. It thus appears likely that the epigenetic basis for this switch evolved to adapt sexual

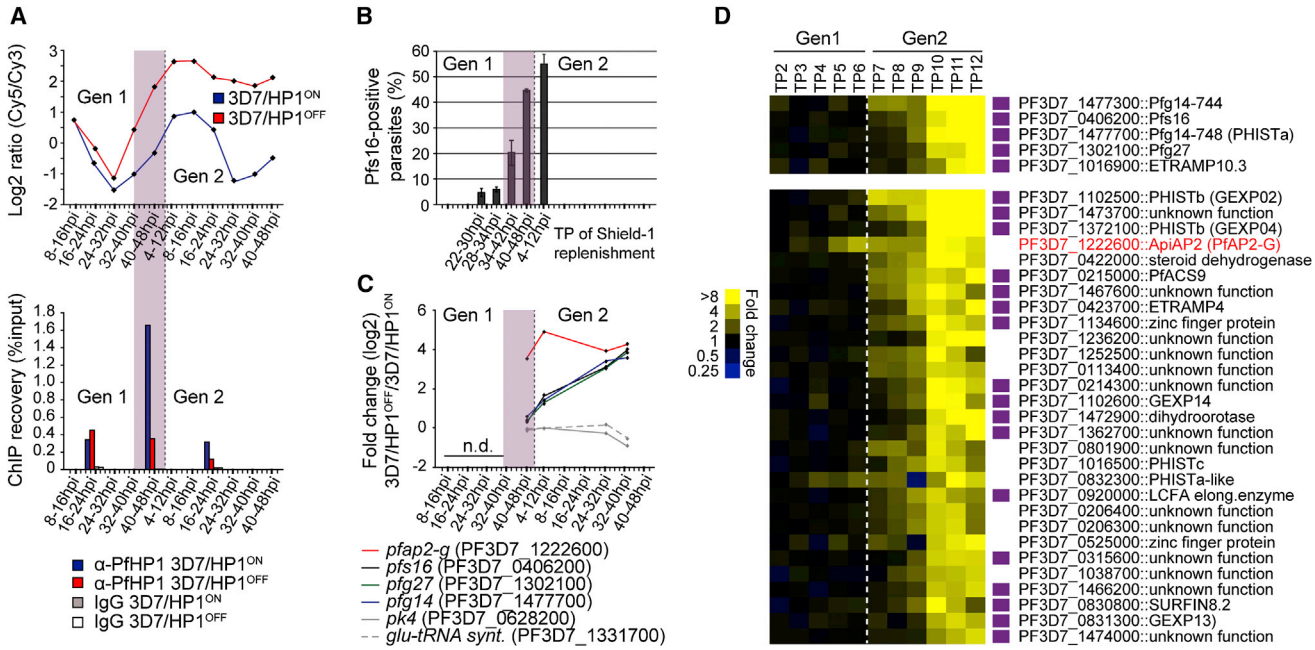

Figure 7. Gametocyte Differentiation Is Linked to the PfHP1-Dependent Activation of *pfap2-g*

(A) Temporal expression profile (Cy5/Cy3 log2 ratios) of *pfap2-g* (top) and ChIP-qPCR results showing PfHP1 occupancy at the *pfap2-g* locus (bottom). The sexual commitment phase is highlighted in purple. See also Figure S6 and Table S1.

(B) Proportion of Pfs16/KAHRP-positive parasites in 3D7/HP1^{OFF} populations rescued at different TPs in generation 1 (x axis) as determined by IFA at 32–40 hr post reinvasion in generation 2. Values show the mean ± SD of three biological replicates. See also Figure S5.

(C) Induction of *pfap2-g* and the three early gametocyte markers *pfs16* (PF3D7_0406200) (Bruce et al., 1994), *pfg27* (PF3D7_1302100) (Alano et al., 1991), and *pfg14_748* (PF3D7_1477700) (Eksi et al., 2005) in 3D7/HP1^{OFF} compared to 3D7/HP1^{ON} parasites as determined by qRT-PCR on biological replicate samples. Negative control genes are in gray. n.d., not determined.

(D) Temporal progression of fold changes in expression of known (upper heatmap) and predicted (lower heatmap) early gametocyte genes in 3D7/HP1^{OFF} compared to 3D7/HP1^{ON} parasites. Early gametocyte genes identified in previous studies are highlighted in purple. See also Figure S7, Table S1, and Supplemental Experimental Procedures.

conversion rates for optimal transmission during the course of infection. Indeed, gametocyte conversion rates are highly variable between different isolates and clones and influenced by a broad spectrum of environmental conditions (Baker, 2010; Alano and Carter, 1990). Although the molecular factor(s) triggering gametocyte conversion have not been identified, two recent studies reported that cell-cell communication via exosomes/microvesicles causes a dramatic increase in gametocyte conversion (Regev-Rudzki et al., 2013; Mantel et al., 2013). It is therefore tempting to speculate that cargo delivered by these vesicles may trigger a signaling cascade that ultimately evicts PfHP1 from the *pfap2-g* locus.

In conclusion, we identified PfHP1 as an essential factor in mitotic proliferation and as a key mediator of two systems of CVGE that secure the survival and transmission of malaria blood-stage parasites, respectively. Our data provide important mechanistic insight into the regulatory processes underlying antigenic variation and sexual conversion and generate knowledge relevant for investigating conceptually similar systems in other eukaryotes. Importantly, we established that gametocyte commitment is epigenetically regulated. This significant discovery will facilitate the targeted dissection of the molecular pathway triggering sexual conversion and has major implications for the identification of approaches to prevent malaria transmission.

EXPERIMENTAL PROCEDURES

Parasite Culture and Transfection

P. falciparum 3D7 cell culture and transfection was performed as described (Trager and Jenson, 1978; Lambros and Vanderberg, 1979; Voss et al., 2006). Transfection constructs are described in the Supplemental Experimental Procedures and in Figure S1. Parasites were grown in the presence of the indicated combinations of 4 nM WR99210 (WR) and 625 nM Shield-1. 3D7/HP1^{ON} clones were obtained by limiting dilution.

Western Blot Analysis

Nuclei were isolated as described (Voss et al., 2003) and lysed in 2% SDS, 10 mM Tris, 1 mM dithiothreitol (pH 8.0). Proteins were detected using rabbit α-PfHP1 1:5,000 (Figure S1 and Supplemental Experimental Procedures) and α-H4 1:10,000 (Abcam ab10158). PfEMP1 was extracted as described (Van Schravendijk et al., 1993) and detected using the pan-specific α-PfEMP1 mouse monoclonal antibody (mAb) 1B/6H-1 (1:500) (Duffy et al., 2002).

Fluorescence Microscopy

Live-cell fluorescence microscopy and IFAs were performed as described (Witmer et al., 2012). IFAs were performed on methanol-fixed cells using mouse immunoglobulin G2a (IgG2a) mAb α-HRP1 (α-KAHRP) (kind gift from D. Taylor), 1:500; mouse IgG1 mAb α-Pfs16 (kind gift from Robert W. Sauerwein), 1:250; mouse IgG1 mAb α-PfEMP1 (1B/6H-1) (Duffy et al., 2002), 1:150; and rabbit α-PfHP1, 1:100. Secondary antibody dilutions were as follows: Alexa Fluor 568-conjugated α-rabbit IgG (Molecular Probes), 1:250; Alexa Fluor 568-conjugated α-mouse IgG2a (Molecular Probes), 1:250; Alexa Fluor 488-conjugated α-mouse IgG1 (Molecular Probes),

1:250; and FITC-conjugated α-mouse IgG (Kirkegaard Perry Laboratories), 1:250. Images were taken at 96-fold magnification on a Leica DM 5000B microscope with a Leica DFC 300 FX camera, acquired via the Leica IM 1000 software, and processed using Adobe Photoshop CS6. For each experiment, images were acquired and processed with identical settings.

Isothermal Microcalorimetry

Isothermal microcalorimetry measures the heat flow produced by a biological sample over time. Isothermal microcalorimetry experiments and data analysis were performed as described using a Thermal Activity Monitor (Model 3102 TAM III, TA Instruments) with minor modifications (Wenzler et al., 2012) (see Supplemental Experimental Procedures).

qRT-PCR

3D7/HP1^{ON} parasites were synchronized twice 16 hr apart to obtain an 8 hr growth window and then split into two populations at 4–12 hpi and cultured in either the presence or absence of Shield-1. 3D7/HP1^{ON} and 3D7/HP1^{OFF} parasites were harvested at 40–48 hpi in generation 1 and at three consecutive time points in generation 2 (4–12 hpi, 24–32 hpi, and 32–40 hpi). 3D7 and F12 populations were synchronized identically, and time points were harvested at 4–12 hpi, 24–32 hpi, and 40–48 hpi. Isolation and processing of total RNA and qRT-PCR were conducted as described with minor modifications (Witmer et al., 2012) (see Supplemental Experimental Procedures). Primer sequences are listed in Table S2.

Targeted Chromatin Immunoprecipitation

3D7/HP1^{ON} parasites were synchronized twice 16 hr apart to obtain an 8 hr growth window and then split into two populations, one of which was taken off Shield-1 at 4–12 hpi. Sample pairs were harvested at 16–24 hpi and 40–48 hpi in generation 1 and at 16–24 hpi in generation 2. Isolation of formaldehyde-crosslinked chromatin and ChIP-qPCR analysis were performed as described with minor modifications (Flueck et al., 2009) using 0.6 μg affinity-purified α-PfHP1, 5 μg α-H3K9me3 (Millipore 07_442), or 5 μg rabbit IgG negative control antibodies (Millipore 12–370) (see Supplemental Experimental Procedures). Primer sequences are listed in Table S2.

Flow Cytometry

Tightly synchronized 3D7/HP1^{ON} parasites were split at 0–4 hpi and cultured in either the presence or absence of Shield-1. At 20–24 hpi after reinvasion, the 3D7/HP1^{ON} and 3D7/HP1^{OFF} populations were synchronized again to obtain a 4 hr growth window. DNA content analysis was carried out on five consecutive TPs in generation 2, starting at 24–28 hpi. Packed RBCs (100 μl) were fixed in 4% formaldehyde/0.015% glutaraldehyde; washed three times in RPMI-HEPES; incubated in 1 ml RPMI-HEPES, 0.1%Triton X-100, 0.1 μg/ml RNase A, and 20 μM FxCycle Far Red stain (Molecular Probes) for 30 min in the dark; and analyzed using an AccuriC6 instrument (BD Biosciences). A minimum of 4,000 gated iRBCs were measured (excitation 640 nm; 30 mW diode; emission detection FL4 675 nm ± 12.5 nm). Acquired data were processed using FlowJo software (Version 10.0.5).

Microarray Experiments and Data Analysis

RNA extraction and cDNA synthesis were carried out as described (Bozdech et al., 2003). Cy5-labeled test cDNAs were hybridized against a Cy3-labeled 3D7 cDNA reference pool that was made by combining total RNA isolated from five consecutive time points across the IDC. Equal amounts of Cy5- and Cy3-labeled samples were hybridized on a *P. falciparum* glass slide microarray containing 10,416 70-mer open reading frame probes (Hu et al., 2007). Hybridization was carried out at 65°C in a MAUI automated hybridization station for at least 12 hr (Bozdech et al., 2003). Slides were washed twice in 0.5× saline-sodium citrate (SSC)/0.02% SDS, once in 0.05× SSC, spun dry, and scanned using the GenePix scanner 4000B and GenePix pro 6.0 software (Axon Laboratory). Detailed protocols describing microarray reannotation, data processing, and analysis are provided in the Supplemental Experimental Procedures section.

ACCESSION NUMBERS

The Gene Expression Omnibus accession number for the microarray data reported in this paper is GSE53176.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, seven figures, and two tables and can be found with this article online at http://dx.doi.org/10.1016/j.chom.2014.07.004.

AUTHOR CONTRIBUTIONS

N.M.B.B. and N.L.B. designed and performed experiments, analyzed data, prepared illustrations, and wrote the paper. W.H.C. performed microarray hybridizations. I.N. expressed recombinant PfHP1 and produced affinity-purified α-PfHP1 antibodies. L.Z. analyzed microarray data. R.W. designed and performed qRT-PCR experiments. C.F. performed isothermal microcalorimetry experiments. M.R., I.F., and Z.B. designed experiments and interpreted data. T.S.V. supervised the study, designed experiments, analyzed data, prepared illustrations, and wrote the manuscript.

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