A Plasmodium falciparum Histone Deacetylase Regulates Antigenic Variation and Gametocyte Conversion

Bradley I. Coleman,1,4 Kristen M. Skillman,1,4 Rays H.Y. Jiang,1,6 Lauren M. Childs,2 Lindsey M. Altenhofen,3,5 Markus Ganter,1 Yvette Leung,1 Ilana Goldowitz,1 Björn F.C. Kafsack,3 Matthias Marti,1 Manuel Llinás,3,5 Caroline O. Buckee,2 and Manoj T. Duraisingh1,*

1Department of Immunology and Infectious Diseases, Harvard School of Public Health, Boston, MA 02115, USA
2Department of Epidemiology and Center for Communicable Disease Dynamics, Harvard School of Public Health, Boston, MA 02115, USA
3Department of Molecular Biology and Lewis-Sigler Institute for Integrative Genomics, Princeton University, Princeton, NJ 08544, USA
4Co-first authors
5Present address: Department of Biochemistry and Molecular Biology and Center for Infectious Disease Dynamics, Pennsylvania State University, State College, PA 16802, USA
6Present address: Department of Global Health and Center for Drug Discovery and Innovation, University of South Florida, Tampa, FL 33612, USA
*Correspondence: mdurai@hsph.harvard.edu

SUMMARY

The asexual forms of the malaria parasite Plasmodium falciparum are adapted for chronic persistence in human red blood cells, continuously evading host immunity using epigenetically regulated antigenic variation of virulence-associated genes. Parasite survival on a population level also requires differentiation into sexual forms, an obligatory step for further human transmission. We reveal that the essential nuclear gene, P. falciparum histone deacetylase 2 (PfHda2), is a global silencer of virulence gene expression and controls the frequency of switching from the asexual cycle to sexual development. PfHda2 depletion leads to dysregulated expression of both virulence-associated var genes and PfAP2-g, a transcription factor controlling sexual conversion, and is accompanied by increases in gametocytogenesis. Mathematical modeling further indicates that PfHda2 has likely evolved to optimize the parasite’s infectious period by achieving low frequencies of virulence gene expression switching and sexual conversion. This common regulation of cellular transcriptional programs mechanistically links parasite transmissibility and virulence.

INTRODUCTION

Eukaryotic pathogens often encounter a trade-off between the establishment of infection within a host and transmission to subsequent hosts. In diverse pathogens, persistence relies on clonal phenotypic variation associated with epigenetically regulated low-frequency stochastic switches (Verstrepen and Fink, 2009). Persistence of Plasmodium falciparum, the most clinically significant cause of human malaria, within the human bloodstream is largely due to phenotypic switching between polymorphic members of the ~60 member var gene family that encodes for the PfEMP1 (P. falciparum Erythrocyte Membrane Protein 1) cytoadherence protein. This process of antigenic variation allows for sequestration of infected red blood cells in the microvasculature, thereby preventing clearance by the spleen (Baruch et al., 1995; Smith et al., 1995; Su et al., 1995).

Switches in expression of var genes and other virulence-associated, clonally variant multigene families are in part mediated by the presence of distinct histone modifications and variants (Lopez-Rubio et al., 2007, 2009; Petter et al., 2011). Active or silent chromatin states at individual loci within P. falciparum are written and maintained by the concerted actions of histone-modifying enzymes such as the class III sirtuin histone deacetylases (HDACs) PISir2a and PISir2b (DuraiSingh et al., 2005; Freitas-Junior et al., 2005; Tonkin et al., 2009) and the histone methyltransferases PISET10 and PISET2 (also known as PISETvs) (Jiang et al., 2013; Volz et al., 2012). HDACs promote transcriptional silencing by removing acetyl groups on histones. This facilitates H3K9 methylation (H3K9me3), P. falciparum heterochromatin protein 1 (PfHP1) binding (Flueck et al., 2009; Lopez-Rubio et al., 2009; Pérez-Toledo et al., 2009), heterochromatin formation, and reduced accessibility for transcription (Coleman et al., 2012; Duraisingh et al., 2005; Scherf et al., 1998).

In addition to control of adhesive phenotypes and immune evasion in eukaryotic pathogens, epigenetic regulation has been shown to have a role in developmental transitions (Saksouk et al., 2005; Sonda et al., 2010). In each asexual cycle of the P. falciparum blood stage, a small subpopulation of parasites converts to the sexual gametocyte form required for transmission to the mosquito vector. Recently, genetic determinants for sexual differentiation have been identified in P. falciparum (Eksi et al., 2012; Ikadai et al., 2013; Rovira-Graells et al., 2012; Silvestrini et al., 2010; Young et al., 2005). However, the molecular basis for the frequency of switching from the asexual cycle to sexual development remains obscure. Unlike bacterial and viral pathogens, where pathogen load directly correlates with the probability of transmission, P. falciparum relies upon an antigendically distinct, avirulent gametocyte form that makes up only a small fraction of parasites in the blood. Indeed, from an evolutionary
perspective it remains unclear why so few transmission stages are produced (Mideo and Day, 2008; Pollitt et al., 2011).

Here, we demonstrate that the class II HDAC protein, \textit{P. falciparum} histone deacetylase 2 (PfHda2), is essential for asexual proliferation in vitro. Through conditional depletion, we identify a role for PfHda2 both as a global regulator of virulence gene expression and as a regulator of the frequency of \textit{P. falciparum} gametocyte conversion, with transcriptional activation predominantly at heterochromatin-enriched loci. These data, in combination with mathematical modeling, suggest that PfHda2 is a critical component of shared epigenetic regulatory machinery that has evolved to provide low-frequency switch rates for both antigenic variation and transmission gene expression programs that enhance the duration of parasite infectiousness.

\section*{RESULTS}

\subsection*{\textit{P. falciparum} Hda2 Is an Essential Nuclear Class II Histone Deacetylase}

We bioinformatically identified PF3D7\_1008000 as one of two >250 kDa putative histone deacetylases (HDACs) within the \textit{P. falciparum} genome. PF3D7\_1008000 contained both of the predictive sequences of a class II enzyme, RPPGHH and LEGGY (catalytic residues in bold) (Figure 1A, and see Figure S1A available online) (Grozinger et al., 1999) and was renamed PfHda2. In addition to the HDAC domain, PfHda2 contains an inositol polyphosphate multikinase (IPMK) domain at its C terminus. IPMKs sequentially generate IP4 and IP5 from the soluble second messenger IP3 (Nalaskowski et al., 2002). This combination of HDAC and IPMK domains is unique to the alveolate phylum including other \textit{Plasmodium} spp, \textit{Toxoplasma}, \textit{Theileria}, \textit{Paramecium}, and \textit{Tetrahymena} (Figure S1B; Table S1). PF3D7\_1472200, the second large HDAC identified, is another class II enzyme that was named PfHda1, although it lacks any homology with PfHda2 outside the HDAC core.

To characterize the timing of its expression and localization, immunofluorescence assays were performed against endogenously epitope-tagged PfHda2 (Figure S1C). Concentrated foci of PfHda2 localized near the nuclear periphery, a largely heterochromatic subcompartment that has been linked to both the activation and silencing of clonally variant genes (Coleman et al., 2012; Duraisingh et al., 2005; Dzikowski et al., 2007; A

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure1}
\caption{PfHda2 Is an Essential Nuclear Histone Deacetylase}
\end{figure}

(A) Schematic of PF3D7\_1008000 protein highlighting HDAC and IPMK domains. Light gray, low-complexity sequence interrupting the HDAC domain. Box, class II HDAC motifs with catalytic residues in bold. Pf, \textit{Plasmodium falciparum}; Pk, \textit{Plasmodium knowlesi}; Pb, \textit{Plasmodium berghei}; Tg, \textit{Toxoplasma gondii}; Sc, \textit{Saccharomyces cerevisiae}. See also Figure S1.

(B) Immunofluorescence detection of PfHda2-HA in the perinuclear region of late stage asexual \textit{P. falciparum}. Scale bar, 3 \(\mu\m)um.

(C) Cellular fractionation of PfHda2-HA parasites to separate the nuclear (N) and cytoplasmic (C) compartments. Histone H3-nuclear control, PfLDH-cytoplasmic control.

(D) Immunofluorescence did not detect PfHda2-HA in merozoites costained with anti-GAP45.
Depletion of PfHda2 Impairs *P. falciparum* Proliferation In Vitro

To determine the cellular function of PfHda2, we attempted to directly disrupt the gene. PfHda2 null parasites were not generated despite multiple attempts using single and double homologous recombination approaches (Figure S2A). The failure of multiple approaches to disrupt the *pfhda2* locus strongly suggests the full-length protein is essential in the asexual cycle of *P. falciparum*.

Given the essential nature of PfHda2, we targeted the C terminus of the endogenous gene with a destabilization domain (DD) for conditional protein expression (Figures S2B and S2C) (Armstrong and Goldberg, 2007). Removing Shield-1 (*Shld1*) from a synchronous culture of early ring stage PfHda2-DD parasites caused a ~95% depletion in PfHda2 protein levels in schizont stage cultures (Figure 2A). Parasite proliferation was unaffected in the first cycle post knockdown but decreased 2-fold in each successive cycle due to a defect in intracellular growth (Figures 2B, 2C, and S2D). When egress and invasion were assayed, knockdown schizonts transitioned to rings with an efficiency similar to that of wild-type (Figures S2E and S2F). DNA content analysis confirmed knockdown parasites progressed normally until a DNA replication defect emerged in a subpopulation of schizonts during S phase in the second cycle following knockdown (Figures S2E and S2G). Thus, PfHda2 depletion profoundly impacts the parasite's ability to proliferate.

**PfHda2 Regulates Expression of Heterochromatic Genes**

To determine the impact of PfHda2 knockdown on *P. falciparum* gene expression, DNA microarrays were used to examine global transcripts at 6 hr intervals from the schizont stage of the first asexual cycle to the end of the second cycle. While the majority of genes were unaffected by PfHda2 knockdown, ~3% of the genome showed a differential expression greater than 2-fold (top 1.5% with Z score >3.4; top 1.5%~3% with Z score >1.6) (Figure 3A). An additional 7% of genes showed a 1.7-fold differential expression (top 3%~8% with Z score >1) (Figure 3A; Table S2). An independent nonparametric estimate showed a similar fraction of differentially expressed genes (11%) (Wilcoxon signed rank-sum test, p < 0.05). Progression through the transcriptional cascade was nearly identical (r > 0.8 Pearson correlation) in wild-type and PfHda2 knockout parasites until 86 hr postinvasion and remained high (r > 0.7 Pearson correlation) for the remainder of the cycle. Transcriptional changes observed prior to 86 hr are therefore not consequences of changes in growth. Dysregulation of gene expression was largely limited to two distinct classes of genes: multigene families exhibiting variant expression throughout the asexual cycle and genes associated with gametocytogenesis (Figure 3A; Table S2) (Eki et al., 2012; Ikada et al., 2013; Rovira-Graells et al., 2012; Silvestrini et al., 2010; Young et al., 2003).

Transcriptional silencing of members of multigene families has been shown to correlate with regions of facultative heterochromatin and the H3K9me3-binding heterochromatin protein HPHP1, while H3K9 acetylation (H3K9ac) marks active genes (Flueck et al., 2009; Lopez-Rubio et al., 2009). We find that 69% of genes dysregulated following PfHda2 knockdown are also bound by HPHP1 (Figures 3B, S3A, and S3B; Table S3) (Flueck et al., 2009), and colocalization of PfHda2-HA and HPHP1 was observed by immunofluorescence assay (Figure 3C). Additionally, PfHda2-regulated genes were significantly H3K9me3 enriched and largely lacked H3K9ac (Figures S3C and S3D) (Salcedo-Amaya et al., 2009). PfHda2 knockdown affected neither HPHP1 localization nor its total protein abundance at this level of resolution (Figures S3E and S3F), nor did it lead to global changes in histone acetylation (Figure S3G), suggesting a highly targeted role for PfHda2 in the mobilization of HPHP1-bound heterochromatin.

**PfHda2 Is a Global Regulator of var Gene Expression**

We identified specific gene families overrepresented in the transcriptional change data set following PfHda2 knockdown (Figure 4A). The most highly changed gene class was the var multigene family (Figure 4A). Of the family, 86% was represented among the most differentially expressed genes, indicating that PfHda2 is a global regulator of var gene silencing. Other multigene families were also dysregulated, including *Pfmc-2TM* (73%) and *rif/stevor* (46%) (Figure 4A).
phenotype (Figure S4C).

delay, similar to that observed for the initial knockdown growth
reversed the parasites’ growth defect, but only after a one-cycle
changes represent bona fide activation of previously silent
H3K9me3 as determined by ChIP, implying that the observed
loci was associated with increased H3K9Ac and decreased
promoters (Figure S4B).

expression in the wild-type strain, showed the smallest relative
prior to PfHda2 knockdown. Conversely, upsA, with the greatest
expression, likely due to this class having the tightest silencing
telomeric subset, demonstrated the greatest fold change in
increase in total
(PfHda2 knockdown but was comparatively slow to turn off
(Figure S4A).

We used the conditional knockdown strain to more closely
study the quantitative expression of the entire var gene repertoire
(Salanti et al., 2003). We observed a universal loss of transcriptional repression across all classes with an overall ~8-fold increase in total var transcripts (Figure 4B). UpsB, the most sub-
telomeric subset, demonstrated the greatest fold change in var expression, likely due to this class having the tightest silencing prior to PfHda2 knockdown. Conversely, upsA, with the greatest expression in the wild-type strain, showed the smallest relative change. As expected, transcriptional activation at specific var loci was associated with increased H3K9Ac and decreased H3K9me3 as determined by ChIP, implying that the observed changes represent bona fide activation of previously silent var promoters (Figure S4B).

To determine whether dysregulation of var expression is reversible within the asexual mitotic cell cycle or requires passage through the insect for meiosis, we used the DD system to revert the PfHda2 protein knockdown by returning Shld1 to the parasite culture. Rescue of the PfHda2 knockdown resulted in a return of silencing across all var classes, demonstrating the reversibility of PfHda2-mediated epigenetic regulation of this process (Figure 4B). Rescue of the PfHda2 knockdown also reversed the parasites’ growth defect, but only after a one-cycle delay, similar to that observed for the initial knockdown growth phenotype (Figure S4C).

Telomere length has previously been associated with changes in var gene expression following the deletion of PISir2a histone deacetylase (Tonkin et al., 2009). We find no telomere-associated changes after a single cycle of PfHda2 knockdown, implying that var expression is not directly linked to telomere length (Figure S4D). After 2 months of PfHda2 knockdown, however, changes in telomere length were observed, suggesting PfHda2 may play a role in the long-term maintenance of chromosome structure (Figure S4D).

PfHda2 Depletion Leads to Increased Gametocyte Conversion

In addition to an impact on multigene families encoding virulence genes, PfHda2 knockdown also dysregulated genes associated with gametocytogenesis (Figure 4A). Nearly 40% of our defined set of sexual-stage gametocyte genes (Eksi et al., 2012; Ikada et al., 2013; Rovira-Graells et al., 2012; Silvestrini et al., 2010; Young et al., 2005) were associated with HP1-bound heterochromatin, and >70% were dysregulated following PfHda2 knockdown (Figures 5A and S5A). Of the heterochromatin-marked gametocyte genes, the most strongly dysregulated gene following PfHda2 knockdown was PF3D7_1222600 (pfap2-g) (Figure S5A), encoding the PfApiAP2 transcription factor shown recently to be required for the transcriptional switch to gametocytogenesis (Kafsack et al., 2014; Sinha et al., 2014). Additionally, the heterochromatinized genes (Pfge2, Pfge7, Pfge8) on chromosome 14 undergo a 1.7-fold or greater differential expression. Other genes are differentially expressed in the PfHda2 knockdown but are not heterochromatin marked, suggesting they may be downstream targets of PfApi2-g. The effect on global var gene dysregulation we find with the PfHda2 knockdown resembles that seen with the methyltransferase PISET2 knockout (Jiang et al., 2013); however, in stark contrast, dysregulation of gametocyte gene expression is unique to PfHda2 knockdown (Figure S5B). Supporting this difference, var loci are enriched with H3K36me3, the histone modification made
Hda2 Regulation of Plasmodium Gene Expression

Figure 4. PfHda2 Knockdown Leads to Global Activation of var Genes
(A) The gene families with the strongest dysregulation (var, rif, stever, Pfmc-2TM, and genes associated with gametocytogenesis [Eksi et al., 2012; Ikadai et al., 2013; Rovira-Graells et al., 2012; Silvestrini et al., 2010; Young et al., 2005]) are compared to a control set of ortholog genes conserved across the apicomplexan species (ApiOrthologs). Individual gene representatives are shown (log2 R/G). r = Pearson correlation coefficients comparing PfHda2 knockdown and wild-type at each time point.

(B) Global var transcription following PfHda2 knockdown (upper and middle panels). Upregulation of var transcription following PfHda2 knockdown is reversible following readdition of Shld1 (bottom panel). Pie charts sized to the total summation of RCN contain slices proportional to expression of each transcript. Data shown are from one representative experiment of three (+/− Shld1) or two (reversion) biological replicates that yielded similar results. Inset, PfHda2-DD protein expression was restored upon readdition of Shld1 after one cycle of knockdown. See also Figure S4.

To determine whether PfHda2 knockdown leads to an increase in the phenotypic rate of conversion to gametocytes, we measured gametocyte production in PfHda2 wild-type and knockdown parasite cultures. We observed a 3-fold increase in the conversion rate in knockdown parasites (Figures 5B, S5D, and S5E). This increase in conversion rate was partially reversed upon readdition of Shld1 to ring stage cultures for two cycles following one cycle of depletion (Figure S5F). In contrast to the var genes, we observed no significant changes in histone modification at the pfap2-g locus following knockdown, consistent with a switch to activation of this locus within only a subset of parasites (Figure S5C). Given the expression of pfhda2 in S phase and pfap2-g in rings, our data support a role for PfHda2 in passing on an epigenetic signature for the expression of pfap2-g in the next cycle (Figure S5C), explaining the observation that commitment to gametocytogenesis occurs in the previous asexual cycle (Eksi et al., 2012).

Shared Epigenetic Regulation of Antigenic Variation and Gametocyte Conversion Optimizes Parasite Infectious Period

Our findings have two significant implications for the understanding of the evolution of P. falciparum malaria parasites. First, although an evolutionary trade-off between virulence and transmission has been postulated for a wide range of pathogen systems, PfHda2 provides a mechanistic link between the expression of malaria virulence factors and the production of transmission stages in malaria parasites. Second, a variety of hypotheses have been put forward to explain the evolution of "reproductive restraint" associated with the paucity of P. falciparum transmission stages in the blood (Mideo and Day, 2008; Pollitt et al., 2011). Using a mathematical model, which allows us to examine the possible in vivo implications of our findings, we explored the parasite dynamics within the host when shared epigenetic machinery controls rates for both antigenic variation and gametocyte conversion. We find that low, but nonzero, switch rates of var gene expression maximize the length of infection, with the duration of infectiousness to
anopheline mosquitoes showing a similar relationship but at marginally higher switch rates (Figure 6A). When var switching rates are too rapid, the parasite quickly presents most of its antigenic variants to the immune system, reducing the duration of infection. However, if the var switching rate is close to zero, the parasite will be unable to evade the immune response and be cleared without the production of sufficient gametocytes to ensure transmission (Figure 6A). In contrast, because the expansion of the asexual population during each round of replication is large enough that rapid proliferation can continue even with substantial rates of switching to transmission stages, the infectious period is comparatively insensitive to increases in gametocyte conversion rate. In combination with our experimental results, therefore, our findings suggest that investment in transmission stages is inextricably linked to the control of antigenic variation, and that reproductive restraint may in fact represent a byproduct of adaptation for chronicity via antigenic variation (Figure 6B).

DISCUSSION

The variegated expression associated with epigenetic regulation is exquisitely suited for the low frequency switches in gene expression underlying antigenic variation, a process required by many eukaryotic pathogens for persistence in hosts (Domergue et al., 2005; Duraisingh et al., 2005; Freitas-Junior et al., 2005; Prucca et al., 2008; Rudenko et al., 1995). Here, we demonstrate that an epigenetic regulator, the class II histone deacetylase PfHda2, is essential for blood-stage proliferation of *P. falciparum*. Employing a conditional protein expression strategy, we find a critical role for this regulator in two fundamental parasitic processes that exhibit clonal variation: antigenic switching and gametocyte conversion.

Variant expression of the var family of virulence genes is epigenetically regulated by several nonessential histone modifying proteins including the sirtuin histone deacetylases, PfSir2a and PfSir2b, which appear to impart a “division of labor” by regulating specific var gene subsets (Duraisingh et al., 2005; Tonkin et al., 2009). This differs from PfHda2, which, like the histone methyltransferase PfSET2 (Jiang et al., 2013), is a global regulator of the var gene family. Depletion of PfHda2 also affects the transcription of other non-var genes that exhibit clonal variation, including a dramatic upregulation of members of the mc-2TM family (Lavazec et al., 2007). The majority of genes in this altered set were marked with PfHP1, likely defining a fundamental genetic circuit wherein PfHda2 nucleates heterochromatin formation (Figure 6B). Similarly, in fission yeast the class II HDAC Clr3 is required for the nucleation and maintenance of heterochromatin at centromeres and the mating locus (Yamada et al., 2005).

Strikingly, unlike PiSET2 and the sirtuins, we find that PfHda2 regulates gametocyte gene expression. The ApiAP2 transcription factor PfAP2-g has recently been identified as a critical switch for transcription of gametocyte genes (Kafsack et al., 2014; Sinha et al., 2014), and this gene becomes activated following depletion of PfHda2. Previous work has mapped the time of gametocyte commitment to the schizont stage in the asexual cell cycle preceding the formation of gametocytes (Bruce et al., 1990; Eksi et al., 2012). This commitment period is consistent with PfHda2 peak expression in the late trophozoite and schizont stages, which include the DNA synthesis phase of the asexual cycle (Figure 5C). This contrasts with PiHP1, which is expressed constitutively through the asexual cycle (Flueck et al., 2009). Indeed, the PfHda2 ortholog in the evolutionarily related alveolate parasite *Tetrahymena thermophila* deacetylates newly synthesized histones (Smith et al., 2008), and fission yeast Clr3
Our model indicates that long infections with low sexual conversion rates provide optimal transmission potential (Figure 6A). We propose that the low rates of gametocyte conversion observed in P. falciparum evolved as a consequence of the shared epigenetic machinery with var switching, which drives these distinct phenotypic switches.

With little selective disadvantage to small variations in gametocyte conversion rates, the epigenetic frequencies may have been principally determined by the strong selective pressure for antigenic variation. Unlike the disparate collection of multi-gene families employed by Plasmodium spp. during asexual development, the core machinery for regulating the production of transmission stages in all Plasmodia, such as PIAp2-g, appears conserved. If the genetic mechanism controlling gametocyte conversion rates evolved prior to regulators of var switch rates, then reproductive restraint may not be adaptive for sexual conversion per se but driven by the need to optimize low switch rates for antigenic variation. These low switch rates (∼1% per generation) may therefore represent a particular strategy for P. falciparum chronicity in areas where mosquito vector availability is seasonal or unreliable. The resulting low levels of gametocyte conversion and transmission may themselves represent exploitation of a biological niche, as, at the other extreme, several hemosporidians, including hepatocystis and hemoprotozoa, bypass blood-stage schizogony and antigenic variation altogether, and immediately form gametocytes after entering the circulation (Martinsen et al., 2008).

It remains to be seen whether PfHda2 activity is modulated in a physiological setting to translate environmental signals into changes in transcriptional programs for antigenic variation and virulence. Importantly, PfHda2 could provide a mechanistic link between var gene dysregulation and sensing of the host environment in severe disease (Merrick et al., 2012; Warimwe et al., 2012). Sexual conversion rates are also thought to be influenced by host signals such as anemia, reticulocyte concentration, cytokines, and microvesicles (Baker, 2010; Mantel et al., 2013; Regov-Rudzki et al., 2013), and PfHda2 may play a central role in coordinating the frequency of sexual conversion and antigenic variation in response to these signals.

Despite the fact that antigenic variation and sexual conversion are not required for proliferation in vitro, we find that the class II PfHda2 is an essential gene, validating it as a target for pharmaceutical inhibition. However, the exact nature of PfHda2 activity remains to be elucidated. PfHda2 conspicuously contains both protein deacetylase and IPMK domains, an alveolate-specific adaptation that suggests they are functionally linked. Both, or either, of these domains could be responsible for the mobilization of heterochromatin. PfHda2, therefore, has great potential for selective inhibition, and indeed, several HDAC-targeted inhibitors have been identified that are toxic to parasites at low nanomolar concentrations, including FDA-approved HDAC inhibitors (Patel et al., 2009). Future studies will determine which phenotypes result from the function of either of the two domains, or indeed whether PfHda2 is a scaffold for a larger silencing machinery with epigenetic functions.

In summary, we have identified PfHda2 as a key regulator of antigenic variation and the frequency of gametocyte conversion. In a related paper in this issue of Cell Host & Microbe, Brancucci et al. (2014) find that the conditional depletion of PfHP1 also has inhibiting histone turnover for the maintenance of heterochromatin (Aygün et al., 2013).

While a low switch rate between var genes has almost certainly evolved to promote chronicity, it has long been questioned why P. falciparum gametocyte conversion rates are so low. Theoretically, it is difficult to account for the evolution of this “reproductive restraint” (McKenzie and Bossert, 1998; Mideo and Day, 2008; Taylor and Read, 1997). Studies of human infectiousness suggest that low gametocyte densities can be efficiently transmitted to mosquitoes (Bousema and Drakeley, 2011; Churcher et al., 2013). This implies that there is unlikely to be a strong selective disadvantage to moderate or low conversion rates, and
profound effects on var gene expression, gametocyte conversion, and proliferation. This is consistent with our proposition that PfHda2 is an upstream regulator of PfHP1, required for heterochromatin formation and gene silencing. Interplay of PfHda2 with other levels of regulation is expected to be important. For instance, the translational repressor PfPuf2 has been shown to be required for gametocyte conversion while suppressing proliferation (Miao et al., 2010). It has been suggested that introducing control programs may increase malaria parasite virulence in some circumstances (Mackinnon et al., 2008). Sharing of core epigenetic machinery suggests that the selection against one of these fundamental parasitic processes during drug or vaccine interventions might have an unexpected effect on the other, with serious public health implications.

EXPERIMENTAL PROCEDURES

Parasite Culture and Transgenic Parasites

The 3D7 strain of P. falciparum was obtained from the Walter and Eliza Hall Institute (Melbourne, Australia). Parasites were cultured as previously described (Trager and Jensen, 1976). Targeting plasmids were constructed and transfected into P. falciparum 3D7 using single crossover recombination (Duraiasingh et al., 2003).

Southern and Western Blotting with Nuclear Fractionation

Preparation of genomic DNA and Southern blotting was performed as previously described (Dvorin et al., 2010). Telomere Restriction Fragment Southern blots were performed as previously described (Merrick et al., 2010). Western blot lysates were probed with the following primary antibodies: α-bloths were performed as previously described (Miao et al., 2010). It has been suggested that introducing control programs may increase malaria parasite virulence in some circumstances (Mackinnon et al., 2008). Sharing of core epigenetic machinery suggests that the selection against one of these fundamental parasitic processes during drug or vaccine interventions might have an unexpected effect on the other, with serious public health implications.

EXPERIMENTAL PROCEDURES

Parasite Culture and Transgenic Parasites

The 3D7 strain of P. falciparum was obtained from the Walter and Eliza Hall Institute (Melbourne, Australia). Parasites were cultured as previously described (Trager and Jensen, 1976). Targeting plasmids were constructed and transfected into P. falciparum 3D7 using single crossover recombination (Duraiasingh et al., 2003).

Southern and Western Blotting with Nuclear Fractionation

Preparation of genomic DNA and Southern blotting was performed as previously described (Dvorin et al., 2010). Telomere Restriction Fragment Southern blots were performed as previously described (Merrick et al., 2010). Western blot lysates were probed with the following primary antibodies: α-HA (1:1,000) (clone 3F10, Roche Applied Science), α-PF 2013 (1:2,000) (gift of Michael T. Makler), α-PfLDH (1:2,000) (gift of Michael T. Makler), α-HA (1:5,000) (Upstate), α-PfH3 (1:500) (gift of Jean-Claude Douy), α-Acetyl-Lysine (1:1,000) (99441, Cell Signaling), α-H3Ac (1:1,000) (06-599, Millipore), α-H3K9Ac (1:2,000) (07-442, Millipore), α-H3K14Ac (1:1,000) (06-911, Millipore), and α-H3K9Me3 (1:2,000) (07-442, Millipore). A previously described P. falciparum nuclear and cytoplasmic fractionation protocol (Voss et al., 2002) was adapted to assay Hda2-HA nuclear localization.

Immunofluorescence Assays

HA-tagged proteins were visualized as previously described (Bushkin et al., 2010). Costaining of HA-tagged PfHda2 with PHP1 (1:500) (gift of Till Voss) or PIGAP45 (1:3,000) (gift of Julian Rayner) was done as previously described (Flueck et al., 2009) (Supplemental Experimental Procedures).

PfHda2 Knockdown, Proliferation, and Cell-Cycle Time Course Assays

PfHda2-HA-DD parasites were synchronized in at least two consecutive cycles as ring stage parasites with 5% D-sorbitol and washed three times in incomplete media to remove Shld1. Pellets were then resuspended in complete RPMI-1640 and split into two identical cultures. Shld1 was returned to only one culture. Cultures were harvested as schizonts, and western blots were performed as above. To measure proliferation, schizont-stage parasites were purified by MACS column (Milenyi) or Percoll gradient (GE Lifesciences). Parasites were allowed to reinvade for 2–4 hr before sorbitol lysis as described above. Proliferation was assayed as previously described (Dvorin et al., 2010). parasites were allowed to reinvade for 2–4 hr before sorbitol lysis as described above. Proliferation was assayed as previously described (Dvorin et al., 2010). Parasite multiplication rate was calculated as (cycle n parasitemia/cycle n-1 parasitemia). For time course experiments, samples were stained with Sybr Green I every 8 hr and measured by flow cytometry with gating by DNA content or counted by thin smear.

Microarray Analysis

Tightly synchronized PfHda2-DD parasites treated ± Shld1 were grown under shaking conditions. Starting at 44 hr post-Shld1 removal, samples were harvested in Trizol for RNA extraction every 6 hr for 66 hr. DNA microarray analysis of global transcription was performed as previously described (Painter et al., 2013) (Supplemental Experimental Procedures). The data discussed in this publication have been deposited in NCBI’s Gene Expression Omimics (Edgar et al., 2002) and are accessible through GEO Series accession number GSE54806 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE54806).

var Expression Analysis and Chromatin Immunoprecipitation-qPCR of PfHda2 Knockdown Parasites

Highly synchronous parasites were obtained via MACS/sorbitol purification as described above. After one full life cycle, ring-stage parasites were harvested for RNA, which was converted to cDNA and analyzed with specific 3D7 var primers as previously described (Merrick et al., 2010; Salanti et al., 2003). Chromatin immunoprecipitation-qPCR was performed as previously described (Coleman et al., 2012) (Supplemental Experimental Procedures).

Gametocyte Conversion Assays

Tightly synchronized ring stage parasites were treated ± Shld1 at 2.5% parasitemia. Following reinvasion, parasitemia was determined using Sybr Green staining and flow cytometry. Cultures were then treated with heparin to stop subsequent reinvasion and allow monitoring of gametocyte formation. At 96 hr post-reinvasion, gametocytes were counted from triplicate wells by thin smear. Conversion rate was calculated as (gametocytes)/(starting parasitemia). For reversion assays, Shld1 was removed from the culture for one cycle and readded for two subsequent cycles. Gametocytes formed from the initial Shld1 depletion were removed by percoll gradient to avoid obscuring the gametocyte counts.

Modeling

A discrete-time stochastic model introduced by Recker et al. (Recker et al., 2011) of the blood-stage parasite dynamics of a P. falciparum infection was modified to include conversion to gametocytes. Here, asexual parasitemia at each 48 hr time step depends on (1) asexual parasites in the previous time step, (2) growth due to the release of multiple merozoites per parasite, (3) removal by the various components of the immune response, and (4) removal of a small fraction in the conversion process to gametocytes. The total parasite population is subdivided by the antigendependently varying protein expressed, and during each time step the parasites have a small probability of switching the expressed variant. The immune response includes both an innate and an adaptive immune response equivalently applied against all variants and two additional adaptive responses, one variant specific and one crossreactive, that respond to particular subsets of variants. To incorporate the simultaneous epigenetic control of variant switching and gametocyte conversion, these were varied proportionally to determine the effects on infectiousness of their linked epigenetic control (Supplemental Experimental Procedures).

SUPPLEMENTAL INFORMATION

Supplemental Information includes five figures, four tables, two movies, and Supplemental Experimental Procedures and can be found with this article at http://dx.doi.org/10.1016/j.chom.2014.06.014.

AUTHOR CONTRIBUTIONS

B.I.C. and K.M.S. designed and performed experiments, analyzed data, and wrote the manuscript. R.H.Y.J. performed the phylogenetic, microarray, and epigenetic mark analysis. L.M.C. performed the mathematical modeling. L.M.A. performed the microarray, M.G. performed the colocalization immunofluorescence. Y.L. performed the telomere restriction fragment blots. I.G. helped design gametocyte conversion assays. B.F.C.K. provided microarray analysis. M.M. supervised gametocyte conversion assays. M.L. supervised the microarray, C.O.B. supervised the modeling and wrote the manuscript. M.T.D. designed experiments, supervised the study, and wrote the manuscript. All authors edited the manuscript.

ACKNOWLEDGMENTS

We thank Katy Shaw Saliba and Deepali Ravel for aid with this work. Support for this research was provided by a National Science Foundation Graduate Research Fellowship (B.I.C.), NIH T32 ST32HL007574-31 (K.M.S.), American
Heart Association 13POST16850007 (K.M.S.), HHMI fellowship of the Damon Runyon Cancer Research Foundation (B.F.C.K.), NIH/NIAID R21 AI05328 (M.M.), NIH R01 AI076276 with support from the Centre for Quantitative Biology (PSG0M071508 (M.L.)), Award U54GM088558 from the National Institute of General Medical Sciences (L.M.C., O.C.B.), and a Burroughs Wellcome Fund New Investigator in the Pathogenesis of Infectious Diseases Fellowship (M.T.D.).

Received: January 26, 2014 Revised: May 6, 2014 Accepted: June 6, 2014 Published: August 13, 2014

REFERENCES


