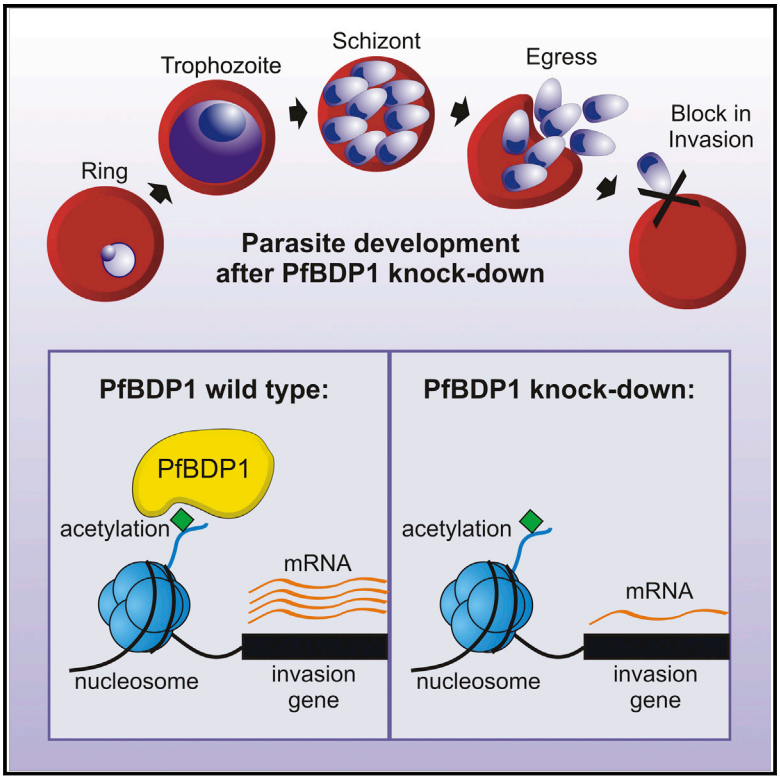
Article

Cell Host & Microbe

A *Plasmodium Falciparum* Bromodomain Protein Regulates Invasion Gene Expression

Graphical Abstract



Authors

Gabrielle A. Josling, Michaela Petter, Sophie C. Oehring, ..., Zbynek Bozdech, Till S. Voss, Michael F. Duffy

Correspondence

till.voss@unibas.ch (T.S.V.), mduffy@unimelb.edu.au (M.F.D.)

In Brief

Invasion of erythrocytes by the human malaria parasite *Plasmodium falciparum* requires the accurate expression of multiple parasite proteins. Josling et al. identify the bromodomain protein PfBDP1 as a critical activator of the coordinate expression of genes that encode proteins with an essential role in erythrocyte invasion.

Highlights

- The bromodomain protein, PfBDP1, is essential in malaria parasites
- PfBDP1 binds to chromatin at invasion gene promoters
- Depletion of PfBDP1 dysregulates invasion gene expression and disrupts invasion

Accession Numbers

GSE64691

Josling et al., 2015, Cell Host & Microbe *17*, 741–751
June 10, 2015
http://dx.doi.org/10.1016/j.chom.2015.05.009

Cell Host & Microbe
Article

A *Plasmodium Falciparum* Bromodomain Protein Regulates Invasion Gene Expression

Gabrielle A. Josling,[1,13,14] Michaela Petter,[1,13] Sophie C. Oehring,[2,3] Archna P. Gupta,[4] Olivier Dietz,[2,3] Danny W. Wilson,[5,6] Thomas Schubert,[7] Gernot Längst,[8] Paul R. Gilson,[9,10] Brendan S. Crabb,[1,9,10] Suzette Moes,[11] Paul Jenoe,[11] Shu Wei Lim,[1] Graham V. Brown,[1,12] Zbynek Bozdech,[4] Till S. Voss,[2,3,*] and Michael F. Duffy[1,*]

[1]Department of Medicine Royal Melbourne Hospital, The Peter Doherty Institute, The University of Melbourne, 792 Elizabeth Street, Melbourne, Victoria 3000, Australia
[2]Department of Medical Parasitology and Infection Biology, Swiss Tropical and Public Health Institute, Socinstrasse 57, Basel 4051, Switzerland
[3]University of Basel, Petersplatz 1, Basel 4003, Switzerland
[4]Nanyang Technological University, School of Biological Sciences, 60 Nanyang Drive, Singapore 637551
[5]School of Molecular and Biomedical Science, The University of Adelaide, South Australia 5005, Australia
[6]Division of Infection and Immunity, Walter and Eliza Hall Institute of Medical Research, Parkville, Victoria 3050, Australia
[7]2bind GmbH, Josef Engert Straße 13, 93053 Regensburg, Germany
[8]Institute for Biochemistry III, University of Regensburg, Universitätsstraße 31, 93053 Regensburg, Germany
[9]Macfarlane Burnet Institute for Medical Research and Public Health, Melbourne, Victoria 3004, Australia
[10]Monash University, Melbourne, Victoria 3800, Australia
[11]Biozentrum, University of Basel, Klingelbergstrasse 50/70, Basel 4056, Switzerland
[12]Nossal Institute for Global Health, The University of Melbourne, 161 Barry Street, Carlton, Victoria 3010, Australia
[13]Co-first author
[14]Present address: Department of Biochemistry and Molecular Biology, W124 Millennium Science Complex, The Pennsylvania State University, State College, PA 16802, USA
*Correspondence: till.voss@unibas.ch (T.S.V.), mduffy@unimelb.edu.au (M.F.D.)
http://dx.doi.org/10.1016/j.chom.2015.05.009

SUMMARY

During red-blood-cell-stage infection of *Plasmodium falciparum*, the parasite undergoes repeated rounds of replication, egress, and invasion. Erythrocyte invasion involves specific interactions between host cell receptors and parasite ligands and coordinated expression of genes specific to this step of the life cycle. We show that a parasite-specific bromodomain protein, PfBDP1, binds to chromatin at transcriptional start sites of invasion-related genes and directly controls their expression. Conditional PfBDP1 knockdown causes a dramatic defect in parasite invasion and growth and results in transcriptional downregulation of multiple invasion-related genes at a time point critical for invasion. Conversely, PfBDP1 overexpression enhances expression of these same invasion-related genes. PfBDP1 binds to acetylated histone H3 and a second bromodomain protein, PfBDP2, suggesting a potential mechanism for gene recognition and control. Collectively, these findings show that PfBDP1 critically coordinates expression of invasion genes and indicate that targeting PfBDP1 could be an invaluable tool in malaria eradication.

INTRODUCTION

Plasmodium falciparum is the organism responsible for the most lethal form of malaria. Despite the complexity of its life cycle, the symptoms of disease are associated only with the red blood cell stage of the infection (Miller et al., 2002). During this intraerythrocytic developmental cycle (IDC), parasites go through repeated rounds of replication, egress, and reinvasion of red blood cells. Invasion of erythrocytes is thus an essential part of the IDC and is a highly complex process involving multiple steps (Cowman et al., 2012). Though the mechanisms underlying invasion have been well studied, to date little is known about how genes involved in invasion are regulated and how coordinate expression of these genes is achieved.

Chromatin-based regulation of gene expression is known to be important in regulating key pathogenic processes in *P. falciparum* (Duffy et al., 2014). This has been best studied in the *var* multigene family of immunodominant variant antigens (Duraisingh et al., 2005; Freitas-Junior et al., 2005; Petter et al., 2011; Volz et al., 2012). Differences in histone modifications that contribute to chromatin structure are also associated with the transcriptional activity of several invasion genes (Comeaux et al., 2011; Cortés et al., 2007; Crowley et al., 2011; Jiang et al., 2010). Many histone modifications have been mapped across the *P. falciparum* genome (Bártfai et al., 2010; Cui et al., 2007; Gupta et al., 2013; Jiang et al., 2013; Lopez-Rubio et al., 2009; Salcedo-Amaya et al., 2009), and many proteins with predicted histone-binding domains have been identified bioinformatically (Bischoff and Vaquero, 2010). However, very few factors which bind to modified histones have been characterized (Dastidar et al., 2013; Flueck et al., 2009; Pérez-Toledo et al., 2009).

The proteins that bind to histone modifications are essential for creating the functional chromatin state. In *P. falciparum* the H3K9me3 binding chromodomain protein PfHP1 maintains heterochromatin and thus variegated expression of *var* genes and

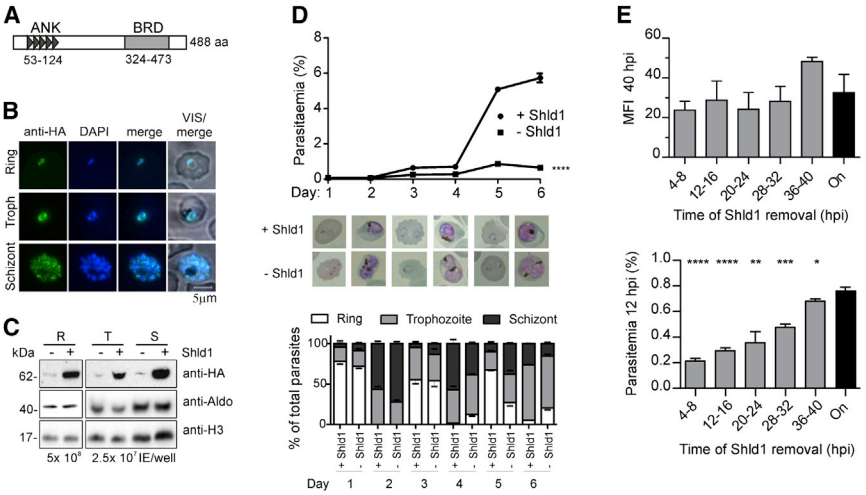


Figure 1. PfBDP1 Is Essential for Parasite Proliferation

(A) The PfBDP1 protein. ANK, Ankyrin repeat; BRD, bromodomain.

(B) Immunofluorescence detection of PfBDP1HA in the nucleus across the asexual life cycle of *P. falciparum*.

(C) Western blot of ring (R, 14 ± 5 hpi), trophozoite (T, 26 ± 5 hpi), and schizont (S, 38 ± 5 hpi) stage PfBDP1DD parasites grown with (+) or without (–) Shld1. Shld1 was removed at 6 ± 5 hpi. See also Figure S1.

(D) Flow cytometry-monitored growth + or – Shld1 of PfBDP1DD parasites expressing GFP (n = 3, bars are SD). Two-way ANOVA (****p < 0.0001). Giemsa-stained blood smears and cumulative parasite stage counts demonstrate normal development of parasites. See also Figure S1.

(E) Effect of Shld1 removal at different time points on growth. MFI, mean fluorescence intensity of IE at 40 hpi. Ring-stage parasitemia was monitored by FACS 12 hpi in the next cycle (n = 3, bars are SD). Student's t test on log10-transformed data (****p < 0.0001, ***p < 0.001, **p < 0.01, *p < 0.05).

also genes critical for regulating gametocytogenesis (Brancucci et al., 2014; Flueck et al., 2009; Pérez-Toledo et al., 2009). In contrast to the silencing role of H3K9me3 and its recruitment of PfHP1, nothing is known about recruitment of activating proteins to *P. falciparum* euchromatin. Acetylated lysines are enriched in histones in transcriptionally inducible euchromatin and are more abundant in *P. falciparum* than in human cells (Trelle et al., 2009). Bromodomain proteins bind acetylated lysines in histones in other eukaryotes and can play a role in gene activation (Josling et al., 2012). *P. falciparum* has seven predicted bromodomain proteins that could potentially bind histone acetylations, but only the conserved histone acetyltransferase GCN5 (Fan et al., 2004) has been characterized.

Here we show that a parasite-specific chromatin-associated factor, bromodomain protein (PfBDP1), directly exerts positive regulation of invasion genes and is required for normal erythrocyte invasion. We find that knockdown of PfBDP1 results in dramatic growth inhibition caused by a defect in erythrocyte invasion, which is accompanied by a significant downregulation of several invasion-related genes in schizonts. We also show that PfBDP1 is enriched at the transcriptional start sites of invasion-related genes in schizonts and that overexpression of PfBDP1 results in their upregulation. These findings also provide a mechanism for coordinated regulation of invasion genes. Therapeutics that inhibited PfBDP1 would disrupt expression of multiple invasion proteins circumventing redundancy in the parasite's repertoire of invasion pathways. Furthermore, PfBDP1 is conserved across apicomplexans, suggesting it could be a therapeutic target in other important apicomplexan parasites of humans and animals.

RESULTS

P. falciparum BDP1 Is an Essential Bromodomain Protein

The bromodomain is the best-known acetyl-lysine binding module, and *P. falciparum* has seven predicted bromodomain proteins, of which five have no known orthologs in higher eukaryotes (Aurrecoechea et al., 2009). One of them, PfBDP1 (PF3D7_1033700), is a 55 kDa protein with an N-terminal ankyrin repeat domain and a single C-terminal bromodomain (Figure 1A), an architecture that is unique to alveolates (Iyer et al., 2008). PfBDP1 is a nuclear protein (Oehring et al., 2012) and is expressed throughout differentiation (Figure 1B and see Figure S4C available online).

In order to investigate the role of PfBDP1 in parasite biology, we created a parasite line in which PfBDP1 was fused at its C terminus to the haemagglutinin (HA) tag and the ligand-regulated FK506-binding protein (FKBP) destabilization domain (DD) to allow for conditional knockdown of the protein (Figure S1). Removing the stabilizing ligand Shld1 resulted in almost complete depletion of PfBDP1DD within 6 hr (Figure 1C). PfBDP1 knockdown parasites had a significantly reduced multiplication rate (2.6 ± 0.7 SD) compared to control parasites (7.1 ± 0.6 SD), although the cells appeared morphologically normal throughout the IDC and no significant difference in staging was apparent for at least the first two cycles (Figures 1D and S1C). Moreover, removal of Shld1 at different time points (TPs) during the IDC resulted in similar nucleic acid content at 40 ± 2 hr postinvasion (hpi) (Figure 1E). PfBDP1 knockdown as late as 36–40 hpi had a significant effect on multiplication, although the phenotype was stronger when the knockdown was induced early in the IDC (Figure 1E).

Depletion of PfBDP1 Impairs Erythrocyte Invasion

Parasites grown in the presence and absence of Shld1 produced equal numbers of merozoites, further suggesting that a defect in schizogony was unlikely to account for the defect in proliferation (Figure 2A). To examine whether the growth phenotype was caused by inhibition of merozoite egress or a block in erythrocyte invasion, we used a fluorescence-activated cell sorting (FACS)-based assay to monitor progression through egress and invasion of PfBDP1 knockdown parasites that were manipulated to also express GFP (Wilson et al., 2013).

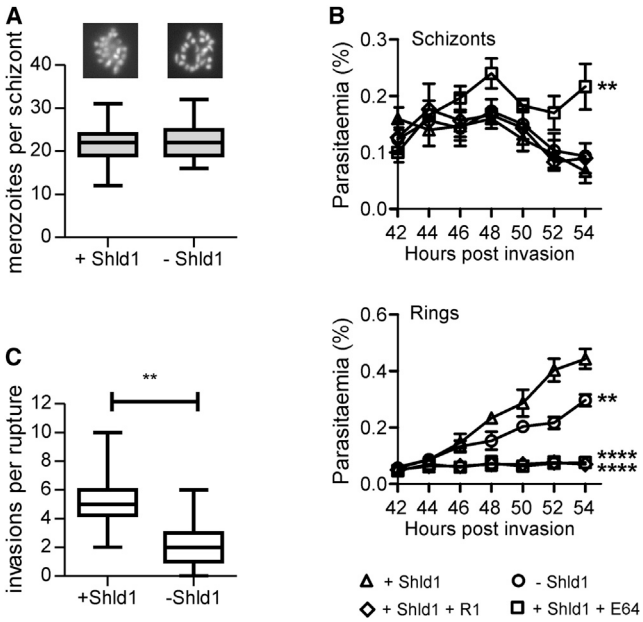


Figure 2. Knockdown of PfBDP1 Results in an Invasion Defect

(A) Number of merozoites per schizont in PfBDP1DD parasites grown + or − Shld1 (duplicate experiments; n = 50 cells for each condition; bars are SD). (Inset) Example fluorescence images of DAPI-stained schizonts.

(B) Flow cytometry growth assay of GFP-expressing PfBDP1DD parasites following removal of Shld1 at 30 hpi. (n = 3, bars are SD). **p < 0.01; ****p < 0.0001, two-way ANOVA. See also Figure S2.

(C) Number of invasions per schizont of PfBDP1DD parasites grown + or − Shld1, determined by video microscopy. Boxes, IQR; whiskers, range; **p < 0.01, Mann-Whitney test. See also Movie S1 and Movie S2.

Parasites grown in the presence or absence of Shld1 were monitored in 2 hr intervals from schizont to ring stage (42–54 hpi; Figures 2B and S2). No accumulation of PfBDP1 knockdown schizonts was observed at any TP, in contrast to control parasites, in which egress was blocked by the cysteine protease inhibitor E64 (Salmon et al., 2001). However, PfBDP1 knockdown parasites did produce significantly fewer ring-stage parasites than parasites grown in the presence of Shld1; the R1 peptide was used as a control to completely block invasion by binding the invasion protein AMA1 (Harris et al., 2005). These experiments demonstrated that PfBDP1 knockdown affects parasite growth primarily by inhibiting invasion while egress occurs normally. Live-cell imaging of rupturing PfBDP1DD schizonts ± Shld1 confirmed that PfBDP1DD schizonts grown in the absence of Shld1 ruptured normally (Movie S1 and Movie S2). However, the number of merozoites per ruptured schizont that invaded successfully was less in the absence of Shld1 (median = 2, n = 8 schizonts) than in the presence of Shld1 (median = 5, n = 16 schizonts) (Figure 2C). Most of the PfBDP1 knockdown merozoites that contacted erythrocytes were able to adhere and deform erythrocytes but were seemingly unable to orientate, penetrate, and trigger echinocytosis (Movie S1). This phenotype suggested defects in a range of mechanisms that could include multiple invasion ligands, the actomyosin motor, and/or signal transduction pathways (Gilson and Crabb, 2009; Sharma and Chitnis, 2013).

PfBDP1 Depletion Results in Deregulation of Invasion Gene Expression

To study the effect of PfBDP1 depletion on gene expression, we performed microarray experiments using RNA harvested at six TPs (8 hr intervals) during the IDC of synchronized PfBDP1DD parasites grown in the presence or absence of Shld1. Global transcription patterns at each TP were similar overall (Figure 3A), but 186 genes were more than 3-fold up- or downregulated at one or more TPs following PfBDP1 depletion (Figure 3B; Table S1). Genes were predominantly upregulated in ring stages and trophozoites (TPs 1–4) and predominantly downregulated during schizogony (TPs 5 and 6) (Figure 3C). Functional enrichment analysis revealed that genes upregulated at TPs 4 (28 ± 4 hpi) and 5 (36 ± 4 hpi) mainly belonged to exported and variant gene families including *rifins* and *pfmc-2tms* that are regulated through heterochromatin (Rovira-Graells et al., 2012) (Figures 3D and S3; Table S2). Strikingly, genes strongly downregulated at TPs 5 and 6 (36 ± 4 and 44 ± 4 hpi) included mainly genes associated with invasion and motility during invasion (Figures 3D and S3; Table S2). In fact, nearly all of the 75 loci annotated as genes encoding merozoite-invasion-related proteins had some level of downregulation at TP 5 (Figure 3E), and 23 showed more than 3-fold downregulation in at least one TP in the IDC. These data strongly support a role for PfBDP1 in transcriptional regulation, particularly of invasion-related genes, and suggest that the invasion defect observed in PfBDP1 knockdown parasites is caused by the disturbed expression of invasion ligands.

Overexpression of PfBDP1 Supports a Role in Invasion Gene Regulation

To validate the effect of PfBDP1 on gene expression, we performed microarrays using RNA isolated from a parasite line overexpressing a PfBDP1-3xHA fusion protein (PfBDP1 OE) (Oehring et al., 2012) and a control line (3D7/cam) (Witmer et al., 2012). RNA was prepared at four TPs during the IDC to identify genes that showed altered expression in response to PfBDP1 overexpression. We confirmed *pfbdp1* overexpression at all four TPs (Figure 4A). In addition, we found that 205 other genes were more than three fold up- or downregulated at any stage compared to the control parasites (Figure 4B; Table S3). In line with our previous findings, invasion-related genes were significantly overrepresented among the genes upregulated in parasites overexpressing PfBDP1 (Figure 4C; Table S4). Strikingly, the majority of invasion-related genes that were downregulated in PfBDP1 knockdown schizonts were upregulated in PfBDP1-overexpressing schizonts (Figure 4D). In general, the changes in gene expression in PfBDP1-overexpressing schizonts were the inverse of those observed in PfBDP1 knockdown schizonts (Figure 4E). Together, these data strongly suggest that PfBDP1 positively regulates genes involved in invasion and explain why PfBDP1 knockdown results in a dramatic invasion defect.

PfBDP1 Acts at the Promoter of Invasion Genes

To address whether PfBDP1 directly regulates invasion genes, we generated the transgenic parasite line PfBDP1HA that expresses PfBDP1 as a HA-tagged fusion protein from the endogeneous promoter (Figure S4) for use in chromatin immunoprecipitation (ChIP). Chromatin was immunoprecipitated from synchronized trophozoite- and schizont-stage parasites and

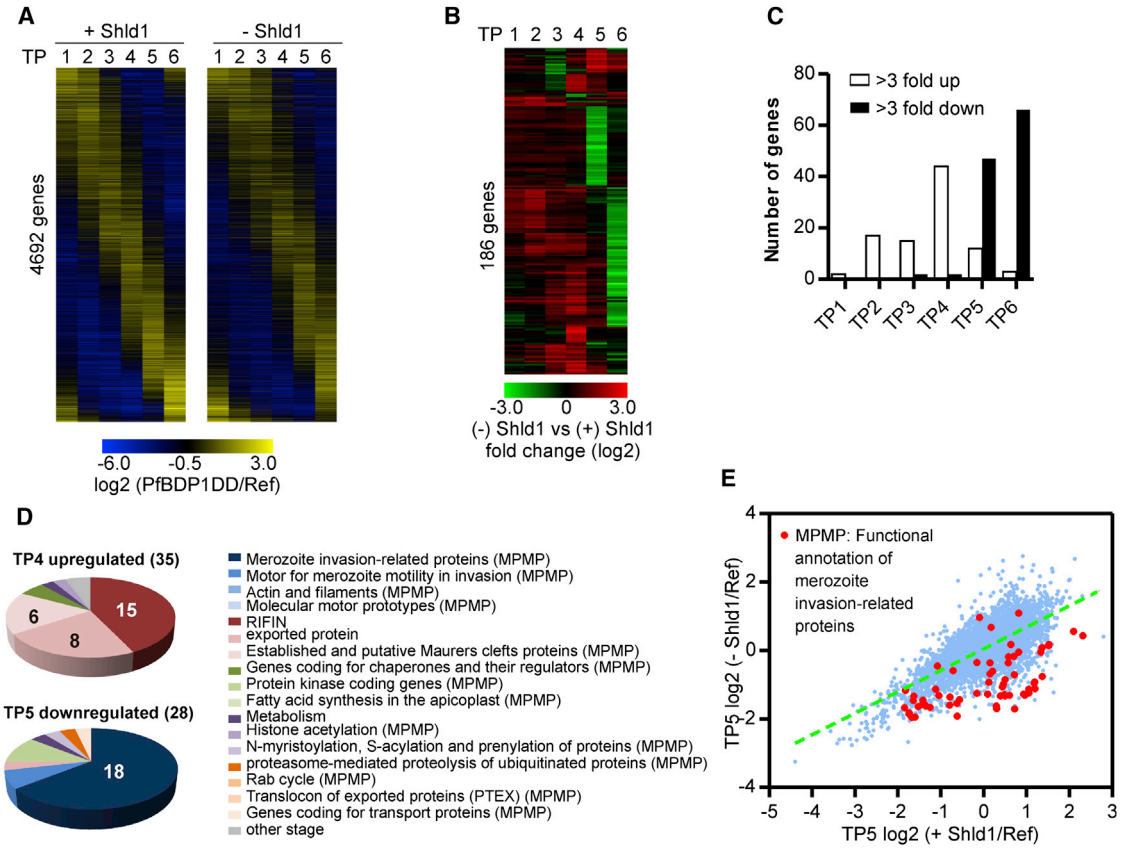


Figure 3. PfBDP1 Knockdown Affects the Expression of Genes Involved in Invasion

(A) Expression-phase-sorted microarrays of PfBDP1DD parasites grown + or − Shld1 across six time points (TPs). Shld1 was removed at 30 hpi in the previous cycle. See also Table S1.

(B) Expression fold change (log2) (− versus + Shld1) across the six TPs for genes induced or repressed more than 3-fold in at least one TP.

(C) Number of genes up- or downregulated more than 3-fold at each TP (q value < 0.05) determined by significance analysis of microarrays (SAM).

(D) Proportions of genes in gene families or malaria parasite metabolic pathways (MPMP) that were more than 3-fold deregulated. See also Table S2.

(E) Gene expression (log2 ratio) in PfBDP1DD schizonts grown + or − Shld1 (TP5). All invasion-related genes are red dots. See also Figure S3.

analyzed by genome-wide sequencing, and specificity was confirmed by qPCR (Figures 5, S4G, and S4H).

Across the genome, 573 genes in schizonts and 95 genes in trophozoites were identified as being PfBDP1 enriched, i.e., they overlapped, or were the closest gene to, PfBDP1 peaks (Table S5). In both stages, PfBDP1-enriched genes were significantly downregulated in response to PfBDP1 knockdown (schizont $p < 0.0001$, trophozoites $p < 0.0184$, Wilcoxon matched-pairs signed rank test) (Figures 5A, 5F, and S4D). Several functional classes of genes were enriched in PfBDP1; importantly, these included 50 genes annotated as playing a role in invasion which were enriched upstream in PfBDP1 (Figures 5C and 5E; Table S5), thus confirming that PfBDP1 directly regulates invasion genes. By contrast, PfBDP1 was not enriched upstream of genes that were upregulated in response to PfBDP1 knockdown (Figures 5F and S4F). These genes may be regulated indirectly by PfBDP1; consistent with this, PfBDP1 was found upstream of seven genes encoding ApiAP2 putative transcription factors (Table S5). In general, genes which were downstream of a PfBDP1 peak were expressed at higher levels than those which were not ($p < 0.0001$ schizonts, $p = 0.0016$ trophozoites, Mann Whitney) (Figures 5B, 5D, S4E, and S4F), providing further evidence that PfBDP1 positively regulates transcription. The expression-associated upstream enrichment of PfBDP1 in schizonts (Figure 5D) was also stage specific, as it was much reduced at the same genes in trophozoites (Figures 5C and S4F). Intriguingly, peaks found upstream of genes in schizonts were enriched in a motif ($p = 2.2e{-}27$, Fisher's exact test, DREME) with homology to predicted binding sites for two ApiAP2 putative specific transcription factors (STFs), including one which has previously been identified upstream of genes encoding rhoptry proteins and merozoite surface proteins involved in invasion (Figure 5G) (Young et al., 2008).

PfBDP1 Binds to Acetylated Histone H3

The association of PfBDP1 with chromatin, its nuclear distribution, and its predicted bromodomain suggested that it regulates gene expression by binding to histones. To test this, we performed a far-western experiment and probed acid-extracted parasite histones with a recombinant glutathione S-transferase (GST)-tagged PfBDP1 bromodomain protein. As a control, we used PfHP1GST, which is known to bind histone 3 trimethylated

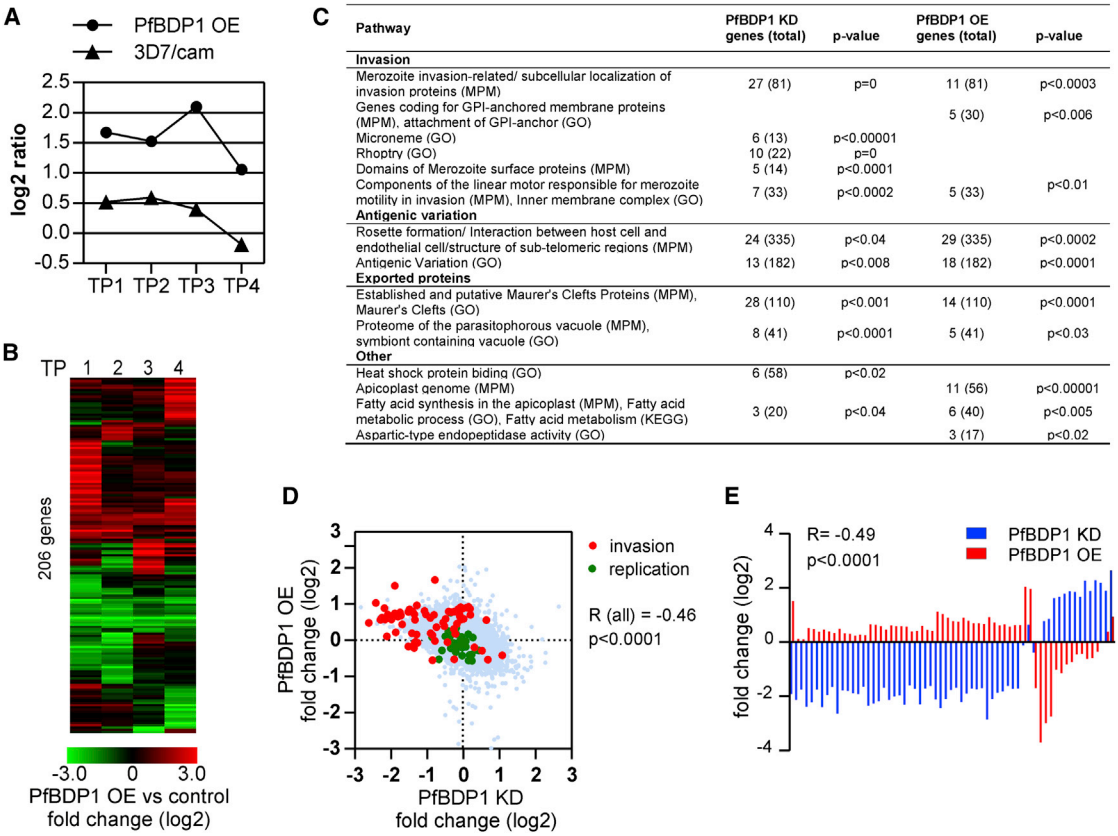


Figure 4. Microarray Analysis Shows that PfBDP1 Overexpression Also Affects Genes Involved in Invasion

(A) PfBDP1 OE overexpresses *pfbdp1* across the IDC relative to control parasites (two-way ANOVA, p = 0.0043). See also Table S3.

(B) Expression fold change (log2) at four TPs for genes induced or repressed more than 3-fold in at least one TP in PfBDP1 OE parasites compared to 3D7/cam control parasites.

(C) Gene function analysis of genes deregulated after PfBDP1 knockdown (KD) or overexpression (OE). See also Table S4.

(D) Expression fold changes (log2) of all genes at schizont stage in PfBDP1 KD (−) Shld1 versus (+) Shld1 are plotted against PfBDP1 OE versus 3D7/cam. Red dots, invasion genes; green dots, replication-related genes as unaffected control.

(E) Expression fold change (log2) (PfBDP1 KD (−) Shld1 versus (+) Shld1 and PfBDP1 OE versus 3D7/cam) of genes (bars) more than 3-fold deregulated in schizonts after either PfBDP1 KD or OE (paired t test).

at lysine 9 (H3K9me3) (Flueck et al., 2009). The bromodomain of PfBDP1 bound to multiple histones, but was predominantly associated with H3 (Figure 6A). Microscale Thermophoresis (MST) experiments further demonstrated that the PfBDP1 bromodomain binds to H3K9ac (Kd = 110.79 ± 10.31 μM [R^2 = 0.995]) and H3K14ac (K_d = 126.17 ± 14.39 μM [R^2 = 0.993]) preferentially over unmodified H3 and H3K16ac, and does not interact with H3K4me2 and H3K9me2 (Figure 6B). Importantly, the binding affinities for H3K9ac and H3K14ac are well within the range of those reported for other bromodomain-histone tail interactions (Filippakopoulos and Knapp, 2012; Filippakopoulos et al., 2012). By ChIPseq we observed partial colocalization between PfBDP1 and H3K9ac at genes enriched upstream in PfBDP1 (Figure S5D). However, the enrichment was no greater than for all other genes, and H3K9ac would be expected at these highly expressed genes independent of PfBDP1 (Salcedo-Amaya et al., 2009). This suggests that H3K9ac is not the sole target of PfBDP1 but might contribute to a multiacetylated binding target (Filippakopoulos et al., 2012).

PfBDP1 Interacts with a Second Bromodomain Protein

Bromodomain proteins generally function as part of larger complexes, where they act to recruit factors that can influence chromatin structure (Josling et al., 2012). To identify PfBDP1-interacting proteins, we immunoprecipitated native PfBDP1-3xHA complexes from PfBDP1 OE parasite nuclear extracts and identified binding partners by liquid chromatography-tandem mass spectrometry (LC-MS/MS) (Table 1; Figure S5; Table S6). Interestingly, another bromodomain protein (Pf3D7_1212900, PfBDP2) was identified with high confidence. Among the other proteins also identified were three previously experimentally localized in the nucleus including a nuclear peroxiredoxin and the RNA/DNA binding protein PfAlba4 (Chêne et al., 2012; Oehring et al., 2012; Richard et al., 2011), and four ribosomal proteins (Table 1). To validate the association of the two bromodomain proteins, we performed coimmunoprecipitation experiments in a double-transfected cell line expressing PfBDP1-3xHA and PfBDP2-2xTy simultaneously. This experiment confirmed the association of PfBDP1 and PfBDP2 (Figure 6C).

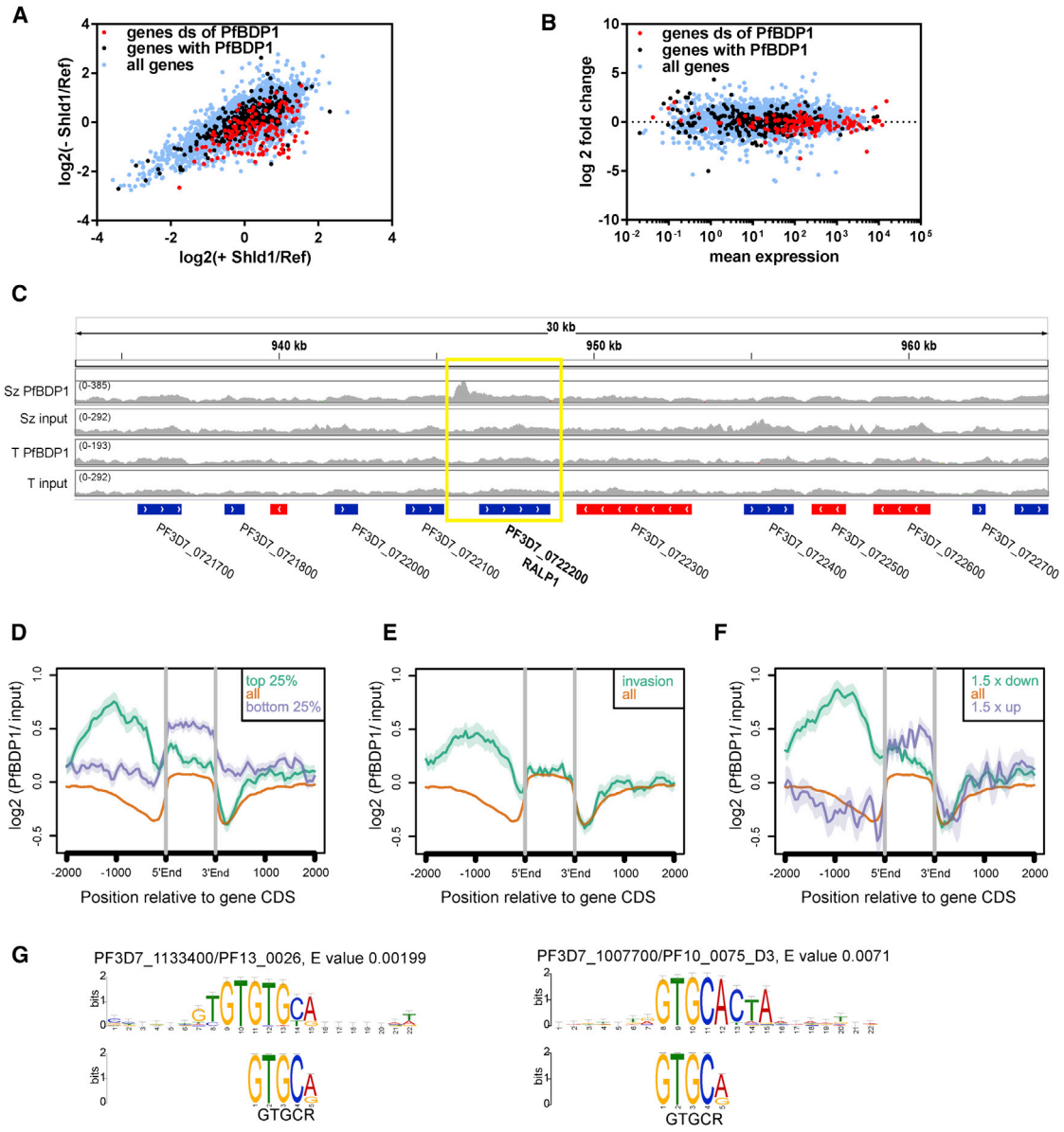


Figure 5. Genome-wide ChIPseq Shows PfBDP1 Binds to Nucleosomes Upstream of Invasion Genes

(A) Scatterplot of schizont stage (TP5) expression microarray data of PfBDP1DD parasites grown + or − Shld1. All genes, all genes closest to (with) PfBDP1, or closest to and downstream (ds) of PfBDP1 peaks are indicated.

(B) MA plot of two replicate schizont RNaseq libraries. All genes, all genes closest to (with) PfBDP1, or closest to and downstream (ds) of PfBDP1 peaks are indicated.

(C) Normalized read coverage plot of reads mapped to a region of chromosome 7 highlighting a PfBDP1 peak upstream of the invasion-related gene *ralp1*.

(D) Average profile of PfBDP1HA enrichment in schizonts (log2 [PfBDP1 ChIP-normalized read coverage/input-normalized read coverage]) relative to gene coding sequences (CDS) for all genes (all); or genes in the top (top 25%, n = 143) or bottom (bottom 25%, n = 143) expression quartile of PfBDP1-enriched genes in schizonts. Shaded regions indicate SEM.

(E) Average profile of PfBDP1HA enrichment in schizonts relative to gene CDS for all genes (all), or genes functionally related to invasion (invasion), n = 119.

(F) Average profile of PfBDP1HA enrichment in schizonts relative to gene CDS for all genes (all); or PfBDP1 enriched genes showing at least 1.5 fold down-regulation (1.5× down, n = 110) or upregulation (1.5-fold up, n = 33) after PfBDP1 knockdown.

(G) DREME logos for a motif enriched within schizont-stage PfBDP1 peaks upstream of genes and the matching published ApiAP2 binding sites identified by Tomtom.

See also Figure S4.

DISCUSSION

Our data strongly support a model in which PfBDP1 directly regulates correct expression of invasion genes. Video microscopy of invading merozoites demonstrated that knockdown of PfBDP1 leads to an invasion defect, explaining the decreased growth observed. A role of PfBDP1 in the transcriptional regulation of invasion genes was confirmed by microarray analysis of

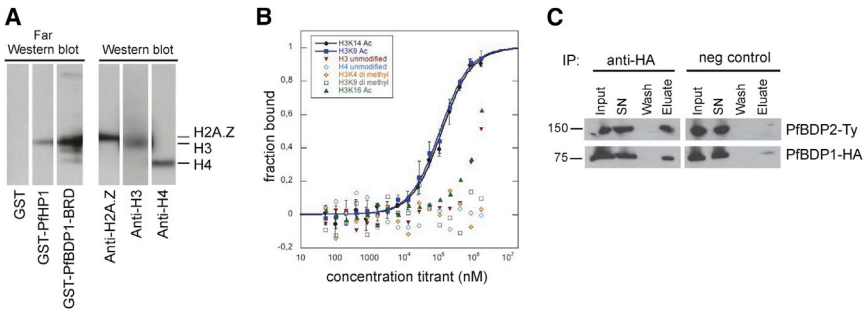


Figure 6. PfBDP1 Interacts with Acetylated Histone 3 and a Protein Complex Including Another Bromodomain Protein

(A) Parasite histones probed with antibodies against histones (western blot) or with recombinant GST-PfBDP1-bromodomain (BRD) or GST-PfHP1 and then anti-GST (far-western blot). GST served as negative control.

(B) MST shows preferential binding of 6xHIS-PfBDP1 to H3K9ac and H3K14ac. Error bars are SD, n = 3.

(C) Input, supernatant (SN), wash, and proteins coimmunoprecipitated with PfBDP1-3xHA (eluate) from parasites coexpressing PfBDP1-3xHA and PfBDP2-2xTY were probed with anti-HA and anti-TY. Excess competing HA peptides blocked binding of PfBDP1-3xHA (negative control).

See also Figure S5.

RNA from parasites in which PfBDP1 was either depleted or overexpressed, which showed that invasion genes were strongly dysregulated. Furthermore, ChIP of PfBDP1 indicated that PfBDP1 is enriched upstream of invasion genes in schizonts, suggesting that it directly regulates these genes. Many of the invasion genes which PfBDP1 regulates are refractory to deletion (Sanders et al., 2006; Triglia et al., 2000) and have been shown to be essential for invasion and thus parasite growth. For example, inhibiting binding of either MSP1 or AMA1 prevents invasion (Harris et al., 2005; Richard et al., 2010; Woehlbier et al., 2010). The dysregulation of some of these genes in response to PfBDP1 knockdown is thus the likely mechanism by which PfBDP1 knockdown results in a defect in invasion. PfBDP1 knockdown also caused a slight delay in gene expression; however, this did not detectably hinder parasite maturation, schizogony, or egress and thus does not detract from the clear evidence that PfBDP1 directly regulates the correct expression of invasion genes.

The stage-specific enrichment of PfBDP1 near the transcriptional start sites of invasion genes in schizonts, but not in trophozoites, suggests that PfBDP1 positively regulates these genes. This is similar to bromodomain proteins in other organisms, which are generally involved in gene activation (Durant and Pugh, 2007; Ng et al., 2002; Peterson and Workman, 2000). Interestingly, perturbation of PfBDP1 levels caused genes to be both downregulated and upregulated. The latter, which included many *rif* genes in trophozoites, were likely indirectly affected by PfBDP1, as it was not enriched upstream of these genes (Figure S4F). This indirect effect could be due to PfBDP1 directly regulating transcriptional repressors. Our data did not implicate a specific repressor, but PfBDP1 was enriched upstream of seven ApiAP2 putative STFs including PfSIP2, which probably plays a role in heterochromatin formation (Flueck et al., 2010) and which was downregulated 2.9-fold after PfBDP1 KD.

Bromodomain proteins often function as part of larger complexes, where they can regulate transcription through a variety of mechanisms. These include recruiting chromatin-remodeling complexes which modify or evict nucleosomes to facilitate transcription, as does Sth1 in *S. cerevisiae* (Parnell et al., 2008), recruiting, or activating RNA polymerase II and general transcription or elongation factors like mammalian Brd4 (Jang et al., 2005; Yang et al., 2005), or directly recruiting STFs like mammalian CBP (Mujtaba et al., 2004). The coimmunoprecipitation of PfBDP1 with several known nuclear proteins suggests that it, too, may be part of a complex. Intriguingly, a second parasite-specific bromodomain protein (PfBDP2) coimmunoprecipitated with PfBDP1. Chromatin-remodeling complexes can contain multiple bromodomain proteins, such as SAGA and RSC (Josling et al., 2012), and our findings similarly suggest that PfBDP1 and PfBDP2 may function within a complex, perhaps to facilitate targeting to certain combinations of histone modifications. Though the only predicted domain in the 125 kDa protein PfBDP2 is a bromodomain, it is possible that it has other, as-yet-uncharacterized functional domains.

A long-standing conundrum of *P. falciparum* nuclear biology is the parasite's ability to tightly regulate the cyclical expression of the majority of its genes despite its limited repertoire of STFs, including the ApiAP2s (Balaji et al., 2005; Bozdech et al., 2003; De Silva et al., 2008). The coordinate activation of multiple invasion genes by PfBDP1 helps resolve this mystery. PfBDP1could act at invasion gene promoters as a coactivator for STFs. For example, the bromodomain protein coactivator ANCCA directly binds the STF E2F1 and recruits it to different target promoters by also binding H3K14ac (Revenko et al., 2010). Indeed, we identified a motif in sites enriched for PfBDP1 in schizonts that showed close resemblance to the ApiAP2 PF3D7_1007700/PF10_0075 binding motif which has previously been identified as enriched upstream of invasion genes (Campbell et al., 2010; Young et al., 2008).

The discovery of an essential parasite-specific bromodomain protein is particularly exciting in light of the emergence of bromodomain proteins as credible drug targets. A bromodomain inhibitor was described in 2005 (Zeng et al., 2005), and recently the therapeutic applications for bromodomain inhibitors have greatly increased. For example, the small molecule JQ1 shows antitumor activity in several cancer types (Delmore et al., 2011; Filippakopoulos et al., 2010; Mertz et al., 2011). Though many of the bromodomain inhibitors which have been developed target BET bromodomains, a class not found in malaria parasites, there are also a number of inhibitors that target other types of bromodomains (Fedorov et al., 2014; Ferguson et al., 2013; Zeng et al., 2005). Parasite-specific bromodomain proteins like PfBDP1 are thus promising drug targets.

Table 1. Potential PfBDP1-Interacting Proteins Identified by LC-MS/MS in CoIP Eluates from Two Independent Experiments

Gene ID	Annotation	Reference Confirmed Nuclear	Percent Sequencecoverage (1)	Percent Sequencecoverage (2)	Number Unique Peptides (1)	Number Unique Peptides (2)
PF3D7_1033700	bromodomain protein, putative (BDP1) (= bait)	Oehring et al., 2012	29.51	39.55	13	14
PF3D7_1212900	bromodomain protein, putative (BDP2)	Oehring et al., 2012	1.1	15.26	1	17
PF3D7_0917900	heat shock protein 70 (HSP70-2)		10.43		5	
PF3D7_1124300	conserved Plasmodium protein, unknown function	Oehring et al., 2012		9.71		3
PF3D7_0517000	60S ribosomal protein L12, putative		9.09	9.09	1	1
PF3D7_0422400	40S ribosomal protein S19, putative		8.24		1	
PF3D7_0507100	60S ribosomal protein L4, putative		3.65		1	
PF3D7_0613800	transcription factor with AP2 domain(s) (ApiAP2)			0.63		1
PF3D7_0919100	DnaJ protein, putative		3.24		1	
PF3D7_0929400	high molecular weight rhoptry protein 2 (RhopH2)		1.38		1	
PF3D7_1027300	peroxiredoxin (nPrx)	Richard et al., 2011		3.82		1
PF3D7_1242700	40S ribosomal protein S17, putative			8.76		1
PF3D7_1347500	DNA/RNA-binding protein Alba 4 (ALBA4)	Chêne et al., 2012	3.49		1	

See also Figure S5 and Table S6. Proteins were exclusively detected in the PfBDP1OE coIP and absent in the negative control. Numbers in parentheses indicate separate experiments.

In conclusion, this study identifies a chromatin protein that plays a key role in the coordinate regulation of genes involved in erythrocyte invasion and thus in the proliferation of malaria parasites. Our data strongly indicate that PfBDP1 regulates invasion-related gene expression through binding to acetylated histones present in nucleosomes at regulatory sites. The importance of the bromodomain protein PfBDP1 in regulating parasite growth and the feasibility of bromodomain inhibitors as therapeutics establish PfBDP1 as an exciting potential drug target.

EXPERIMENTAL PROCEDURES

Parasite Culture

Parasites were cultured as described previously (Trager and Jensen, 1976). Transfection constructs are described in Supplemental Experimental Procedures and in Figures S1 and S4. Transfected cell lines were grown in the presence of 2.5 nM WR99210, 0.5 μM Shld1, and/or 1 μg/mL blasticidin, depending on the parasite line. Clones were obtained by limiting dilution.

Flow Cytometry Growth Assays

PfBDP1DD GFP parasites were tightly synchronized to a 4 hr growth window, and Shld1 was removed at 30 hpi and parasites monitored by flow cytometry across three cycles (Figure 1) or across egress and invasion (42–54 hpi) in 2 hr intervals (Figure 2). Parasites were stained with 5 μg/mL ethidium bromide (Life Technologies) in PBS and then analyzed immediately using a CyAn flow cytometer (Beckman Coulter). A 488 nm laser was used for excitation of GFP fluorescent- and ethidium bromide (EtBr)-stained parasites, as appropriate. At least 20,000 red blood cells were counted for each sample. Data were analyzed using Summit 4.3 (Beckman Coulter). Intact red blood cells were gated for by forward scatter and side scatter, and then rings were defined as $GFP^{high}/EtBr^{low}$ cells, trophozoites as $GFP^{high}/EtBr^{medium}$, and schizonts as $GFP^{high}/EtBr^{high}$ cells (Wilson et al., 2013). Parasites not stained with EtBr- and GFP-negative parasites were used as controls to establish appropriate compensation and gating. AMA1-blocking R1 peptide (Harris et al., 2005) was used as control for a block in invasion and E64 (Salmon et al., 2001) as a control for a block in egress. Data were analyzed by two-way ANOVA in GraphPad Prism.

Time-Lapse Imaging

PfBDP1DD ring stage parasites were synchronized with 5% sorbitol two times 4 hr apart and then grown with or without Shld1 until they were late schizonts. The parasites (2 mL) were settled onto a 35 mm Fluorodish (World Precision Instruments) at 0.16% hematocrit and imaged on an inverted Zeiss AxioObserver microscope in brightfield. The sample chamber was heated to 37°C and supplied with a humidified 5% O2, 5% CO2, and 94% N2 atmosphere. Mature schizonts were selected for imaging based on their likelihood of rupturing within several minutes according to the criteria of Crick et al. (2013).

Microarray Experiments

PfBDP1DD parasites synchronized to an 8 hr growth window were split into two cultures at 30 hpi, and Shld1 was removed from one batch. RNA from three biological replicates was harvested in the following cycle at 0–8 hpi (TP1), 8–16 hpi (TP2), 16–24 hpi (TP3), 24–32 hpi (TP4), 32–40 hpi (TP5), and 40–48 hpi (TP6) by lysis in TRIzol (Life Technologies). Growth of PfBDP1 OE and 3D7/cam control parasites was synchronized to a 10 hr window. Total RNA was isolated at four TPs across the IDC at 4–14 hpi, 14–24 hpi, 24–34 hpi, and 34–44 hpi by lysis of pelleted RBCs in TriReagent (Sigma). Total

RNA extraction and cDNA synthesis were carried out as described (Bozdech et al., 2003) and labeled cDNA hybridized to microarrays containing 5,402 50-mer intergenic oligonucleotide probes and 10,416 70-mer open reading frame probes representing 5,343 coding genes (Hu et al., 2007). Data have been deposited in NCBI's Gene Expression Omnibus (GSE64691). Detailed protocols of microarray hybridization and analysis are provided in Supplemental Experimental Procedures.

Genome-wide Chromatin Immunoprecipitation Assay

Crosslinked chromatin was prepared from PfBDP1HA and control 3D7 parasite cultures at trophozoite and schizont stage essentially as described previously (Petter et al., 2013). Chromatin was precipitated with 1 μg of rat mAb anti-HA 3F10 (Roche). Immunoprecipitated material from PfBDP1HA and 3D7 control parasites was analyzed by qPCR to confirm specific enrichment. PfBDP1HA ChIP and input sequencing libraries were analyzed on a HiSeq2500 (Illumina) at the Melbourne Translational Genomics Platform. Detailed protocols for data analysis are provided in Supplemental Experimental Procedures.

Far Western Blot

Recombinant GST fusion proteins of the PfBDP1 bromodomain and full-length PfHP1 were made and purified as described previously (Petter et al., 2011). Far-western blot was conducted as described (Wu et al., 2007) with minor modifications (see Supplemental Experimental Procedures).

Microscale Thermophoresis

Recombinant PfBDP1 bromodomain (aa 303–488) was expressed as a N-terminal 6xHIS fusion protein. MST binding experiments were carried out with varied concentrations of different modified histone tails (Eurogentec, Germany) on a Monolith NT.labelfree at 25°C (NanoTemper Technologies, Germany) (Seidel et al., 2013). H3K14ac and H3K9ac were tested in triplicate (indicated by the error bars); the other ligands were tested in one biological experiment. Detailed protocols for protein expression and MST are provided in Supplemental Experimental Procedures.

Coimmunoprecipitation and Capillary Liquid Chromatography Tandem Mass Spectrometry

Nuclear extract from PfBDP1 OE schizonts was incubated with agarose beads coated with mAb anti-HA 3F10 (Roche Diagnostics). As negative control, specific competition using an excess of HA peptides (0.1μg/μl) was applied. Bound proteins were eluted using RIPA buffer supplemented with 0.25 μg/μl or 0.5 μg/μl HA-peptides. Coimmunoprecipitated proteins were analyzed by LC-MS/MS as described (Flueck et al., 2009). MS/MS spectra were searched against a combined *P. falciparum* (http://www.plasmodb.org/plasmo/; release 11.1)/human annotated protein database using Proteome Discoverer 1.4.0 (Thermo Scientific, Reinach, Switzerland) using the two search engines Mascot and SequestHT (Table S5). For the search, oxidized methionine and N-terminal protein acetylation were used as variable modifications. The identifications were filtered to a false discovery rate of 1%. Detailed protocols for nuclear extracts and LC-MS/MS are provided in Supplemental Experimental Procedures.

Coimmunoprecipitation in Double-Transfected Parasites

CoIPs of double-transfected cell lines were performed as above and eluted using 2% SDS, 10 mM Tris-HCl (pH 7.5). Samples were analyzed by western blot using anti-HA 3F10 and anti-Ty BB2 antibodies (kind gift from Keith Gull).

ACCESSION NUMBERS

The Gene Expression Omnibus accession number for the microarray expression, RNAseq, and ChIPseq data reported in this paper is GSE64691.

SUPPLEMENTAL INFORMATION

Supplemental Information includes five figures, two movies, six tables, and Supplemental Experimental Procedures and can be found with this article at http://dx.doi.org/10.1016/j.chom.2015.05.009.

AUTHOR CONTRIBUTIONS

G.A.J. and M.P. designed and performed experiments, analyzed data, and wrote the paper; S.C.O., A.P.G., O.D., D.W.W., T.S., P.R.G., and S.M. all designed and performed experiments and analyzed data; S.W.L. performed experiments; G.V.B. and P.J. provided conceptual advice; B.S.C., P.J., and G.L. provided resources; Z.B. supervised experiments; M.F.D. and T.S.V. conceived the study, designed, supervised, performed, and analyzed experiments, and wrote the paper. All authors contributed to editing of the manuscript.

ACKNOWLEDGMENTS

The authors thank Yen Hoon Luah, Igor Niederwieser and Shamista Selvarajah for technical assistance; Stuart Ralph, Kym Pham, and Mark Bailey for advice; and Christopher Tonkin and Alan Cowman for reagents. This work was supported by ARC grant number DP110100483, the Swiss National Science Foundation (PP00P3_130203), the Novartis Foundation for Medical-Biological Research (08C46), the Emilia-Guggenheim-Schnurr Foundation, OzEMalaR, and the Rudolf Geigy Foundation.

Received: December 22, 2014
Revised: March 30, 2015
Accepted: May 14, 2015
Published: June 10, 2015

REFERENCES

Aurrecoechea, C., Brestelli, J., Brunk, B.P., Dommer, J., Fischer, S., Gajria, B., Gao, X., Gingle, A., Grant, G., Harb, O.S., et al. (2009). PlasmoDB: a functional genomic database for malaria parasites. Nucleic Acids Res. *37* (Database issue), D539–D543.

Balaji, S., Babu, M.M., Iyer, L.M., and Aravind, L. (2005). Discovery of the principal specific transcription factors of Apicomplexa and their implication for the evolution of the AP2-integrase DNA binding domains. Nucleic Acids Res. *33*, 3994–4006.

Bártfai, R., Hoeijmakers, W.A.M., Salcedo-Amaya, A.M., Smits, A.H., Janssen-Megens, E., Kaan, A., Treeck, M., Gilberger, T.W., Françoijs, K.J., and Stunnenberg, H.G. (2010). H2A.Z demarcates intergenic regions of the plasmodium falciparum epigenome that are dynamically marked by H3K9ac and H3K4me3. PLoS Pathog. *6*, e1001223.

Bischoff, E., and Vaquero, C. (2010). In silico and biological survey of transcription-associated proteins implicated in the transcriptional machinery during the erythrocytic development of Plasmodium falciparum. BMC Genomics *11*, 34.

Bozdech, Z., Llinás, M., Pulliam, B.L., Wong, E.D., Zhu, J., and DeRisi, J.L. (2003). The transcriptome of the intraerythrocytic developmental cycle of Plasmodium falciparum. PLoS Biol. *1*, E5.

Brancucci, N.M., Bertschi, N.L., Zhu, L., Niederwieser, I., Chin, W.H., Wampfler, R., Freymond, C., Rottmann, M., Felger, I., Bozdech, Z., and Voss, T.S. (2014). Heterochromatin protein 1 secures survival and transmission of malaria parasites. Cell Host Microbe *16*, 165–176.

Campbell, T.L., De Silva, E.K., Olszewski, K.L., Elemento, O., and Llinás, M. (2010). Identification and genome-wide prediction of DNA binding specificities for the ApiAP2 family of regulators from the malaria parasite. PLoS Pathog. *6*, e1001165.

Chêne, A., Vembar, S.S., Rivière, L., Lopez-Rubio, J.J., Claes, A., Siegel, T.N., Sakamoto, H., Scheidig-Benatar, C., Hernandez-Rivas, R., and Scherf, A. (2012). PfAlbas constitute a new eukaryotic DNA/RNA-binding protein family in malaria parasites. Nucleic Acids Res. *40*, 3066–3077.

Comeaux, C.A., Coleman, B.I., Bei, A.K., Whitehurst, N., and Duraisingh, M.T. (2011). Functional analysis of epigenetic regulation of tandem RhopH1/clag genes reveals a role in Plasmodium falciparum growth. Mol. Microbiol. *80*, 378–390.

Cortés, A., Carret, C., Kaneko, O., Yim Lim, B.Y., Ivens, A., and Holder, A.A. (2007). Epigenetic silencing of Plasmodium falciparum genes linked to erythrocyte invasion. PLoS Pathog. *3*, e107.

Cowman, A.F., Berry, D., and Baum, J. (2012). The cellular and molecular basis for malaria parasite invasion of the human red blood cell. J. Cell Biol. *198*, 961–971.

Crick, A.J., Tiffert, T., Shah, S.M., Kotar, J., Lew, V.L., and Cicuta, P. (2013). An automated live imaging platform for studying merozoite egress-invasion in malaria cultures. Biophys. J. *104*, 997–1005.

Crowley, V.M., Rovira-Graells, N., Ribas de Pouplana, L., and Cortés, A. (2011). Heterochromatin formation in bistable chromatin domains controls the epigenetic repression of clonally variant Plasmodium falciparum genes linked to erythrocyte invasion. Mol. Microbiol. *80*, 391–406.

Cui, L., Miao, J., Furuya, T., Li, X., Su, X.Z., and Cui, L. (2007). PfGCN5-mediated histone H3 acetylation plays a key role in gene expression in Plasmodium falciparum. Eukaryot. Cell *6*, 1219–1227.

Dastidar, E.G., Dzeyk, K., Krijgsveld, J., Malmquist, N.A., Doerig, C., Scherf, A., and Lopez-Rubio, J.J. (2013). Comprehensive histone phosphorylation analysis and identification of Pf14-3-3 protein as a histone H3 phosphorylation reader in malaria parasites. PLoS ONE *8*, e53179.

De Silva, E.K., Gehrke, A.R., Olszewski, K., León, I., Chahal, J.S., Bulyk, M.L., and Llinás, M. (2008). Specific DNA-binding by apicomplexan AP2 transcription factors. Proc. Natl. Acad. Sci. USA *105*, 8393–8398.

Delmore, J.E., Issa, G.C., Lemieux, M.E., Rahl, P.B., Shi, J., Jacobs, H.M., Kastritis, E., Gilpatrick, T., Paranal, R.M., Qi, J., et al. (2011). BET bromodomain inhibition as a therapeutic strategy to target c-Myc. Cell *146*, 904–917.

Duffy, M.F., Selvarajah, S.A., Josling, G.A., and Petter, M. (2014). Epigenetic regulation of the Plasmodium falciparum genome. Brief Funct. Genomics *13*, 203–216.

Duraisingh, M.T., Voss, T.S., Marty, A.J., Duffy, M.F., Good, R.T., Thompson, J.K., Freitas-Junior, L.H., Scherf, A., Crabb, B.S., and Cowman, A.F. (2005). Heterochromatin silencing and locus repositioning linked to regulation of virulence genes in Plasmodium falciparum. Cell *121*, 13–24.

Durant, M., and Pugh, B.F. (2007). NuA4-directed chromatin transactions throughout the Saccharomyces cerevisiae genome. Mol. Cell. Biol. *27*, 5327–5335.

Fan, Q., An, L., and Cui, L. (2004). Plasmodium falciparum histone acetyltransferase, a yeast GCN5 homologue involved in chromatin remodeling. Eukaryot. Cell *3*, 264–276.

Fedorov, O., Lingard, H., Wells, C., Monteiro, O.P., Picaud, S., Keates, T., Yapp, C., Philpott, M., Martin, S.J., Felletar, I., et al. (2014). [1,2,4]triazolo [4,3-a]phthalazines: inhibitors of diverse bromodomains. J. Med. Chem. *57*, 462–476.

Ferguson, F.M., Fedorov, O., Chaikuad, A., Philpott, M., Muniz, J.R., Felletar, I., von Delft, F., Heightman, T., Knapp, S., Abell, C., and Ciulli, A. (2013). Targeting low-druggability bromodomains: fragment based screening and inhibitor design against the BAZ2B bromodomain. J. Med. Chem. *56*, 10183–10187.

Filippakopoulos, P., and Knapp, S. (2012). The bromodomain interaction module. FEBS Lett. *586*, 2692–2704.

Filippakopoulos, P., Qi, J., Picaud, S., Shen, Y., Smith, W.B., Fedorov, O., Morse, E.M., Keates, T., Hickman, T.T., Felletar, I., et al. (2010). Selective inhibition of BET bromodomains. Nature *468*, 1067–1073.

Filippakopoulos, P., Picaud, S., Mangos, M., Keates, T., Lambert, J.P., Barsyte-Lovejoy, D., Felletar, I., Volkmer, R., Müller, S., Pawson, T., et al. (2012). Histone recognition and large-scale structural analysis of the human bromodomain family. Cell *149*, 214–231.

Flueck, C., Bartfai, R., Volz, J., Niederwieser, I., Salcedo-Amaya, A.M., Alako, B.T., Ehlgen, F., Ralph, S.A., Cowman, A.F., Bozdech, Z., et al. (2009). Plasmodium falciparum heterochromatin protein 1 marks genomic loci linked to phenotypic variation of exported virulence factors. PLoS Pathog. *5*, e1000569.

Flueck, C., Bartfai, R., Niederwieser, I., Witmer, K., Alako, B.T., Moes, S., Bozdech, Z., Jenoe, P., Stunnenberg, H.G., and Voss, T.S. (2010). A major role for the Plasmodium falciparum ApiAP2 protein PfSIP2 in chromosome end biology. PLoS Pathog. *6*, e1000784.

Freitas-Junior, L.H., Hernandez-Rivas, R., Ralph, S.A., Montiel-Condado, D., Ruvalcaba-Salazar, O.K., Rojas-Meza, A.P., Mâncio-Silva, L., Leal-Silvestre, R.J., Gontijo, A.M., Shorte, S., and Scherf, A. (2005). Telomeric heterochromatin propagation and histone acetylation control mutually exclusive expression of antigenic variation genes in malaria parasites. Cell *121*, 25–36.

Gilson, P.R., and Crabb, B.S. (2009). Morphology and kinetics of the three distinct phases of red blood cell invasion by Plasmodium falciparum merozoites. Int. J. Parasitol. *39*, 91–96.

Gupta, A.P., Chin, W.H., Zhu, L., Mok, S., Luah, Y.H., Lim, E.H., and Bozdech, Z. (2013). Dynamic epigenetic regulation of gene expression during the life cycle of malaria parasite Plasmodium falciparum. PLoS Pathog. *9*, e1003170.

Harris, K.S., Casey, J.L., Coley, A.M., Masciantonio, R., Sabo, J.K., Keizer, D.W., Lee, E.F., McMahon, A., Norton, R.S., Anders, R.F., and Foley, M. (2005). Binding hot spot for invasion inhibitory molecules on Plasmodium falciparum apical membrane antigen 1. Infect. Immun. *73*, 6981–6989.

Hu, G., Llinás, M., Li, J., Preiser, P.R., and Bozdech, Z. (2007). Selection of long oligonucleotides for gene expression microarrays using weighted rank-sum strategy. BMC Bioinformatics *8*, 350.

Iyer, L.M., Anantharaman, V., Wolf, M.Y., and Aravind, L. (2008). Comparative genomics of transcription factors and chromatin proteins in parasitic protists and other eukaryotes. Int. J. Parasitol. *38*, 1–31.

Jang, M.K., Mochizuki, K., Zhou, M., Jeong, H.S., Brady, J.N., and Ozato, K. (2005). The bromodomain protein Brd4 is a positive regulatory component of P-TEFb and stimulates RNA polymerase II-dependent transcription. Mol. Cell *19*, 523–534.

Jiang, L., López-Barragán, M.J., Jiang, H., Mu, J., Gaur, D., Zhao, K., Felsenfeld, G., and Miller, L.H. (2010). Epigenetic control of the variable expression of a Plasmodium falciparum receptor protein for erythrocyte invasion. Proc. Natl. Acad. Sci. USA *107*, 2224–2229.

Jiang, L., Mu, J., Zhang, Q., Ni, T., Srinivasan, P., Rayavara, K., Yang, W., Turner, L., Lavstsen, T., Theander, T.G., et al. (2013). PfSETvs methylation of histone H3K36 represses virulence genes in Plasmodium falciparum. Nature *499*, 223–227.

Josling, G.A., Selvarajah, S.A., Petter, M., and Duffy, M.F. (2012). The role of bromodomain proteins in regulating gene expression. Genes (Basel) *3*, 320–343.

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

Mertz, J.A., Conery, A.R., Bryant, B.M., Sandy, P., Balasubramanian, S., Mele, D.A., Bergeron, L., and Sims, R.J., 3rd. (2011). Targeting MYC dependence in cancer by inhibiting BET bromodomains. Proc. Natl. Acad. Sci. USA *108*, 16669–16674.

Miller, L.H., Baruch, D.I., Marsh, K., and Doumbo, O.K. (2002). The pathogenic basis of malaria. Nature *415*, 673–679.

Mujtaba, S., He, Y., Zeng, L., Yan, S., Plotnikova, O., Sachchidanand, Sanchez, R., Zeleznik-Le, N.J., Ronai, Z., and Zhou, M.M. (2004). Structural mechanism of the bromodomain of the coactivator CBP in p53 transcriptional activation. Mol. Cell *13*, 251–263.

Ng, H.H., Robert, F., Young, R.A., and Struhl, K. (2002). Genome-wide location and regulated recruitment of the RSC nucleosome-remodeling complex. Genes Dev. *16*, 806–819.

Oehring, S.C., Woodcroft, B.J., Moes, S., Wetzel, J., Dietz, O., Pulfer, A., Dekiwadia, C., Maeser, P., Flueck, C., Witmer, K., et al. (2012). Organellar proteomics reveals hundreds of novel nuclear proteins in the malaria parasite Plasmodium falciparum. Genome Biol. *13*, R108.

Parnell, T.J., Huff, J.T., and Cairns, B.R. (2008). RSC regulates nucleosome positioning at Pol II genes and density at Pol III genes. EMBO J. *27*, 100–110.

Pérez-Toledo, K., Rojas-Meza, A.P., Mancio-Silva, L., Hernández-Cuevas, N.A., Delgadillo, D.M., Vargas, M., Martínez-Calvillo, S., Scherf, A., and Hernandez-Rivas, R. (2009). Plasmodium falciparum heterochromatin protein 1 binds to tri-methylated histone 3 lysine 9 and is linked to mutually exclusive expression of var genes. Nucleic Acids Res. *37*, 2596–2606.

Peterson, C.L., and Workman, J.L. (2000). Promoter targeting and chromatin remodeling by the SWI/SNF complex. Curr. Opin. Genet. Dev. *10*, 187–192.

Petter, M., Lee, C.C., Byrne, T.J., Boysen, K.E., Volz, J., Ralph, S.A., Cowman, A.F., Brown, G.V., and Duffy, M.F. (2011). Expression of P. falciparum var genes involves exchange of the histone variant H2A.Z at the promoter. PLoS Pathog. *7*, e1001292.

Petter, M., Selvarajah, S.A., Lee, C.C., Chin, W.H., Gupta, A.P., Bozdech, Z., Brown, G.V., and Duffy, M.F. (2013). H2A.Z and H2B.Z double-variant nucleosomes define intergenic regions and dynamically occupy var gene promoters in the malaria parasite Plasmodium falciparum. Mol. Microbiol. *87*, 1167–1182.

Revenko, A.S., Kalashnikova, E.V., Gemo, A.T., Zou, J.X., and Chen, H.W. (2010). Chromatin loading of E2F-MLL complex by cancer-associated coregulator ANCCA via reading a specific histone mark. Mol. Cell. Biol. *30*, 5260–5272.

Richard, D., MacRaild, C.A., Riglar, D.T., Chan, J.A., Foley, M., Baum, J., Ralph, S.A., Norton, R.S., and Cowman, A.F. (2010). Interaction between Plasmodium falciparum apical membrane antigen 1 and the rhoptry neck protein complex defines a key step in the erythrocyte invasion process of malaria parasites. J. Biol. Chem. *285*, 14815–14822.

Richard, D., Bartfai, R., Volz, J., Ralph, S.A., Muller, S., Stunnenberg, H.G., and Cowman, A.F. (2011). A genome-wide chromatin-associated nuclear peroxiredoxin from the malaria parasite Plasmodium falciparum. J. Biol. Chem. *286*, 11746–11755.

Rovira-Graells, N., Gupta, A.P., Planet, E., Crowley, V.M., Mok, S., Ribas de Pouplana, L., Preiser, P.R., Bozdech, Z., and Cortés, A. (2012). Transcriptional variation in the malaria parasite Plasmodium falciparum. Genome Res. *22*, 925–938.

Salcedo-Amaya, A.M., van Driel, M.A., Alako, B.T., Trelle, M.B., van den Elzen, A.M.G., Cohen, A.M., Janssen-Megens, E.M., van de Vegte-Bolmer, M., Selzer, R.R., Iniguez, A.L., et al. (2009). Dynamic histone H3 epigenome marking during the intraerythrocytic cycle of Plasmodium falciparum. Proc. Natl. Acad. Sci. USA *106*, 9655–9660.

Salmon, B.L., Oksman, A., and Goldberg, D.E. (2001). Malaria parasite exit from the host erythrocyte: a two-step process requiring extraerythrocytic proteolysis. Proc. Natl. Acad. Sci. USA *98*, 271–276.

Sanders, P.R., Kats, L.M., Drew, D.R., O'Donnell, R.A., O'Neill, M., Maier, A.G., Coppel, R.L., and Crabb, B.S. (2006). A set of glycosylphosphatidyl inositol-anchored membrane proteins of Plasmodium falciparum is refractory to genetic deletion. Infect. Immun. *74*, 4330–4338.

Seidel, S.A., Dijkman, P.M., Lea, W.A., van den Bogaart, G., Jerabek-Willemsen, M., Lazic, A., Joseph, J.S., Srinivasan, P., Baaske, P., Simeonov, A., et al. (2013). Microscale thermophoresis quantifies biomolecular interactions under previously challenging conditions. Methods *59*, 301–315.

Sharma, P., and Chitnis, C.E. (2013). Key molecular events during host cell invasion by Apicomplexan pathogens. Curr. Opin. Microbiol. *16*, 432–437.

Trager, W., and Jensen, J.B. (1976). Human malaria parasites in continuous culture. Science *193*, 673–675.

Trelle, M.B., Salcedo-Amaya, A.M., Cohen, A.M., Stunnenberg, H.G., and Jensen, O.N. (2009). Global histone analysis by mass spectrometry reveals a high content of acetylated lysine residues in the malaria parasite Plasmodium falciparum. J. Proteome Res. *8*, 3439–3450.

Triglia, T., Healer, J., Caruana, S.R., Hodder, A.N., Anders, R.F., Crabb, B.S., and Cowman, A.F. (2000). Apical membrane antigen 1 plays a central role in erythrocyte invasion by Plasmodium species. Mol. Microbiol. *38*, 706–718.

Volz, J.C., Bártfai, R., Petter, M., Langer, C., Josling, G.A., Tsuboi, T., Schwach, F., Baum, J., Rayner, J.C., Stunnenberg, H.G., et al. (2012). PfSET10, a Plasmodium falciparum methyltransferase, maintains the active var gene in a poised state during parasite division. Cell Host Microbe *11*, 7–18.

Wilson, D.W., Langer, C., Goodman, C.D., McFadden, G.I., and Beeson, J.G. (2013). Defining the timing of action of antimalarial drugs against Plasmodium falciparum. Antimicrob. Agents Chemother. *57*, 1455–1467.

Witmer, K., Schmid, C.D., Brancucci, N.M., Luah, Y.H., Preiser, P.R., Bozdech, Z., and Voss, T.S. (2012). Analysis of subtelomeric virulence gene families in Plasmodium falciparum by comparative transcriptional profiling. Mol. Microbiol. *84*, 243–259.

Woehlbier, U., Epp, C., Hackett, F., Blackman, M.J., and Bujard, H. (2010). Antibodies against multiple merozoite surface antigens of the human malaria parasite Plasmodium falciparum inhibit parasite maturation and red blood cell invasion. Malar. J. *9*, 77.

Wu, Y., Li, Q., and Chen, X.Z. (2007). Detecting protein-protein interactions by Far western blotting. Nat. Protoc. *2*, 3278–3284.

Yang, Z., Yik, J.H.N., Chen, R., He, N., Jang, M.K., Ozato, K., and Zhou, Q. (2005). Recruitment of P-TEFb for stimulation of transcriptional elongation by the bromodomain protein Brd4. Mol. Cell *19*, 535–545.

Young, J.A., Johnson, J.R., Benner, C., Yan, S.F., Chen, K., Le Roch, K.G., Zhou, Y., and Winzeler, E.A. (2008). In silico discovery of transcription regulatory elements in Plasmodium falciparum. BMC Genomics *9*, 70.

Zeng, L., Li, J., Muller, M., Yan, S., Mujtaba, S., Pan, C., Wang, Z., and Zhou, M.M. (2005). Selective small molecules blocking HIV-1 Tat and coactivator PCAF association. J. Am. Chem. Soc. *127*, 2376–2377.