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Article

PfSET10, a *Plasmodium falciparum* Methyltransferase, Maintains the Active *var* Gene in a Poised State during Parasite Division

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SUMMARY

A major virulence factor of the malaria parasite *Plasmodium falciparum* is *e*rythrocyte *m*embrane *p*rotein 1 (PfEMP1), a variant protein expressed on the infected erythrocyte surface. PfEMP1 is responsible for adherence of infected erythrocytes to the endothelium and plays an important role in pathogenesis. Mutually exclusive transcription and switched expression of one of 60 *var* genes encoding PfEMP1 in each parasite genome provides a mechanism for antigenic variation. We report the identification of a parasite protein, designated PfSET10, which localizes exclusively to the perinuclear active *var* gene expression site. PfSET10 is a histone 3 lysine 4 methyltransferase required to maintain the active *var* gene in a poised state during division for reactivation in daughter parasites, and as such is required for *P. falciparum* antigenic variation. PfSET10 likely maintains the transcriptionally permissive chromatin environment of the active *var* promoter and thus retains memory for heritable transmission of epigenetic information during parasite division.

INTRODUCTION

Malaria, an important infectious disease of humans, causes clinical cases in 300–500 million people and up to 1 million deaths per year. *Plasmodium falciparum* accounts for most of the burden of infection, and virulence is linked to the ability of infected erythrocytes to sequester in and obstruct the microvasculature of a variety of organs (MacPherson et al., 1985). Sequestration avoids destruction of the parasitized erythrocytes by the reticuloendothelial system and allows the microaerophilic parasite to mature in a relatively hypoxic environment (Raventos-Suarez et al., 1985). Moreover, this often leads to perturbation or complete obstruction of blood flow in the microcirculation. This abnormal circulatory behavior for red blood cells is related to parasite-induced alterations of adhesive properties and is key to the survival and pathogenicity of *P. falciparum* (Leech et al., 1984). Parasite-infected erythrocytes can adhere to platelets, vascular endothelial cells, and other erythrocytes (Barnwell, 1989; Berendt et al., 1989; Cooke and Coppel, 1995; Ockenhouse et al., 1992; Rogerson et al., 1995; Wahlgren et al., 1994).

The major virulence factor of *P. falciparum* is *e*rythrocyte *m*embrane *p*rotein 1 (PfEMP1), a protein expressed by the parasite on the surface of infected erythrocytes (Baruch et al., 1995; Su et al., 1995). This protein is responsible for adherence of *P. falciparum*-infected erythrocytes to receptors on endothelial cells and plays an important role in pathogenesis (Bull et al., 1998; Newbold et al., 1997). PfEMP1 proteins are encoded by approximately 60 *var* genes per genome and are located in subtelomeric and central regions of chromosomes (Gardner et al., 2002). Mutual exclusive transcription and switching to activate a different *var* gene provides the basis for antigenic variation and expression of ligands with differing receptor-binding properties (Baruch et al., 1995; Smith et al., 1995; Su et al., 1995).

The *var* gene repertoire resides at the nuclear periphery in clusters of chromosomes (Freitas-Junior et al., 2000), where activation involves chromatin alterations (Voss et al., 2006) and repositioning of a specific *var* gene to a perinuclear location permissive for transcription (*var* gene expression site) characterized by histone marks H3K4me3 and H3K9ac and the presence of H2.AZ (Duraisingh et al., 2005; Dzikowski et al., 2007; Freitas-Junior et al., 2005; Howitt et al., 2009; Lopez-Rubio et al., 2007; Ralph et al., 2005; Petter et al., 2011), while repressed *var* genes are characterized by the conserved histone mark for heterochromatin, H3K9me3 (Lopez-Rubio et al., 2007).

The *var* gene promoter paired with the promoter found within the *var* intron are key elements in nucleation of transcriptional silencing, activation, and maintenance of allelic *var* gene exclusion (Dzikowski et al., 2006; Voss et al., 2006). The identity and molecular function of nuclear factors regulating chromatin modifications at the *var* promoter is not well understood. Although the *P. falciparum* genome contains a large repertoire of putative chromatin-modifying proteins, to date only a handful of nuclear factors have been characterized (Cui and Miao, 2010).

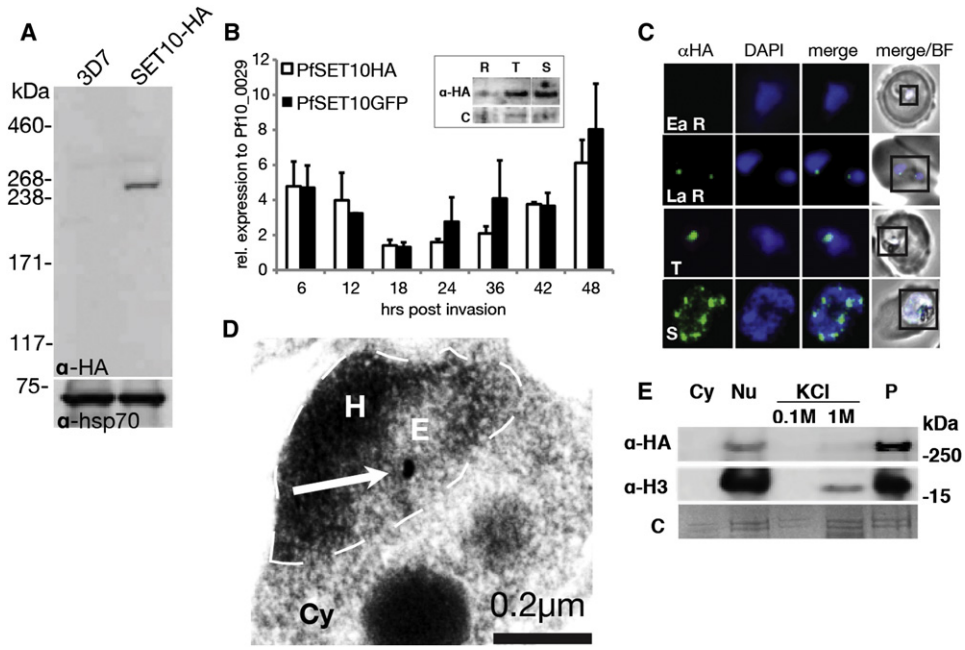

Figure 1. PfSET10 Localizes to a Compartment in the Nuclear Periphery

(A) PfSET10 (PFL1010c) was tagged with 3-HA epitopes, and anti-HA antibodies detected a 260 kDa protein. 3D7 served as control and Hsp70 as a loading control.

(B) Transcription analysis of PfSET10HA and PfSET10GFP in the intraerythrocytic life stages. Small inset shows PfSET10 protein expression in R, rings; T, trophozoites; S, schizonts. Data are represented as mean ± SEM of two independent experiments.

(C) PfSET10-HA was detected using anti-HA antibodies in IFA and shown to localize to a compartment of the nuclear periphery. First row, Ea R early ring; second row, La R, late ring; third row, T, trophozoite; fourth row, S, schizont; each row shows PfSET10 (green); DAPI staining (blue); PfSET10 and DAPI merged; PfSET10, DAPI and brightfield image.

(D) Immunoelectron microscopy localization of PfSET10 in 3D7SET10-HA schizonts using anti-HA antibodies. White arrow points to gold particles localizing with PfSET10. Nucleus is circled by a white dashed line. N, nucleus; Cy, cytoplasm.

(E) PfSET10 is located in the nuclear fraction. Shown are the cytoplasmic (Cy) and nuclear (Nu) fractions probed with anti-HA (upper panel) or anti-H3 antibodies (middle panel). Subsequent tracks show differential solubilisation of the nuclear fraction using 0.1 M and 1 M KCl and the insoluble fraction (P). The bottom panel shows a Coomassie-stained gel as a loading control. See also Figure S1.

This includes two silent information regulators (PfSirA and PfSirB) that play a key role in deacetylation of histone H3 and silencing of *var* genes (Duraisingh et al., 2005; Tonkin et al., 2009). Additionally, *P. falciparum* heterochromatin protein 1 (PfHP1) is a major component of heterochromatin in the perinuclear chromosome end clusters that binds specifically to H3K9me3 (Flueck et al., 2009).

Here, we report identification of a regulator of *var* gene activation in *P. falciparum* named PfSET10. PfSET10 was confined to a location in a euchromatic region of the nuclear periphery corresponding to the active *var* gene expression site. PfSET10 exhibits histone H3 lysine 4 methyltransferase activity, and our data indicate that PfSET10 is responsible for maintaining the *var* gene in the poised stage for re-activation in daughter cells.

RESULTS

*Pf*SET10 Localizes to a Site at the Nuclear Periphery

Previously, we identified a candidate regulator of *var* gene activation in *P. falciparum* (Volz et al., 2010), which we named PfSET10 (PFL1010c) and generated transgenic parasites by tagging the endogenous protein with GFP (Volz et al., 2010) or HA (Figures 1A, 1B and S1A). The HA-tagged PfSET10 had an approximate size of 260 kDa (Figure 1A). PfSET10 is transcribed throughout the intraerythrocytic life cycle, peaking during early and late stages (Figure 1B) in 3D7SET10-HA and 3D7SET10-GFP parasites (Figure 1B). Western analysis showed modest PfSET10 protein expression during ring stage compared to later stages (Figure 1B). This was supported by immunofluorescence assays (IFA) demonstrating no detectable PfSET10 protein in early ring stage but expression in a discrete perinuclear spot in each nucleus of later stage parasites (Figure 1C). Immunoelectron microscopy showed regions of electron-dense material at each nuclear periphery consistent with heterochromatin (Ralph et al., 2005), while PfSET10 localized to a heterochromatin-free region (Figures 1D and S1B). Subcellular fractionation of parasites confirmed PfSET10 was associated with nuclear chromatin (Figure 1E), similar to histone H3.

PfSET10 Localizes to the *var* Gene Expression Site

Current evidence suggests that *var* gene activation involves translocation of the locus to a specific region at the nuclear periphery (Duraisingh et al., 2005; Ralph et al., 2005; Voss et al., 2006; Dzikowski et al., 2007). The presence of PfSET10 in a subcompartment of the perinucleus suggested it could be a regulator of the active *var* in the expression site. We used

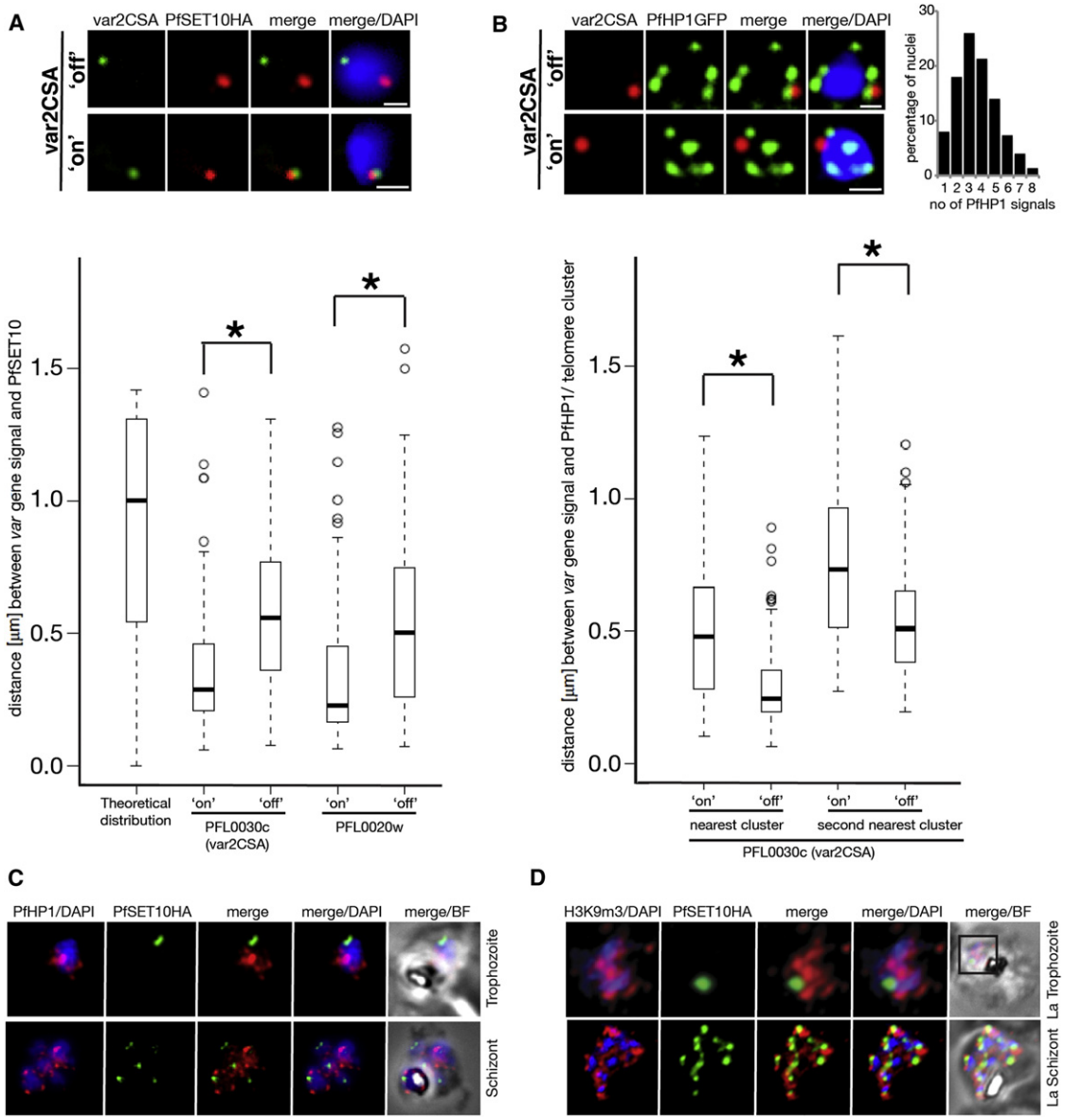

Figure 2. PfSET10 Colocalizes to the Activated *var* Gene

(A) IFA-FISH of 3D7SET10-HA, in which *var2csa* (PFL0030c) activation has been selected by CSA adherence (“on”) (bottom panel). Unselected 3D7SET10-HA (top panel) has *var2csa* “off.” The localization of *var2csa* (green) and PfSET10 (red) is shown with both merged with the nucleus stained by DAPI (blue).Scale bars are equivalent to 0.5 µm. Independently, another *var* gene (PFL0020w) has been selected for activation by ICAM adherence (“on”), and sample distributions (PFL0030c “on” n = 200; “off” n = 173; PFL0020w “on” n = 122; “off” n = 111) are plotted using a standard Tukey boxplot; * stands for $p < 0.000$.

(B) PfHP1 does not localize with active *var2csa* (“on”). The *var2csa* gene (red) location is shown with respect to PfHP1 protein (green). Right panel shows the distribution of PfHP1 signal numbers detected in 150 nuclei. Sample distributions (“on” n = 150; “off” n = 137) are plotted below using a standard Tukey boxplot; * stands for $p < 0.000$.

(C and D) (C) PfHP1 (red) and (D) histone mark H3K9me3 (red,), known markers of silent *var* gene clusters and PfSET10 (green), do not colocalize in the nucleus stained with DAPI (blue). See also Figure S2.

IFA-fluorescent in situ hybridization (IFA-FISH) (Flueck et al., 2009) to determine if PfSET10 colocalized with the active/ poised *var* gene (Figure 2). 3D7SET10-HA parasites were selected for expression of two *var* genes, *var2CSA (*PFL0030c) or PFL0020w (“on”), the genes encoding a *Pf*EMP1 specifying adherence to chondroitin sulfate A (CSA) (Salanti et al., 2003) and *i*ntercellular *a*dhesion *m*olecule-1 (ICAM) (Petter et al., 2011) respectively. As control, we used unselected 3D7SET10-HA (“off”) parasites, in most of which *var2CSA* and PFL0020w were expected to be silent (“off”). Predominant *var2csa* and PFL0020w transcription in 3D7SET10-HA/CSA was confirmed by qRT-PCR (Figure S2A). The analysis was performed in late ring/early trophozoites stages, in which the previously active *var* gene enters the poised state, when PfSET10 was expressed

in a single perinuclear spot. The distance between PfSET10 and *var2csa* and PFL0020w loci was measured to achieve unbiased results (Figures 2A and S2B) and found to be significantly smaller in parasites where the *var* gene was active (''on'') (Kolmogorov-Smirnov test: ''on'' distribution is shifted to smaller distances compared to ''off'', with p value = 0.9952).

To define the position of silenced *var* gene clusters with respect to PfSET10 expression, we used antibodies to PfHP1, a protein localizing with chromosome end clusters and histone mark H3K9me3, which are both associated with silenced *var* gene clusters (Flueck et al., 2009). PfHP1 and the histone mark H3K9me3 showed no overlap with PfSET10 expression (Figures 2C and 2D). The association of PfHP1 with the silent *var* gene was confirmed by IFA-FISH on CSA selected (*var2csa* ''on'') and nonselected (*var2csa* ''off'') 3D7HP1-GFP (Figure 2B). The distance between PfHP1 and the *var2csa* locus was measured to achieve unbiased results (Figures 2B and S2B) and was significantly smaller in parasites where the *var* gene was silent (''off''). Also the distance to the second nearest PfHP1 demarcated telomere cluster was significantly smaller (Figures 2B and S2B). Taken together, these results show that PfSET10 localizes to a perinuclear compartment of the nucleus shared by the active *var* gene.

PfSET10 Is a Histone Lysine 4 Methyltransferase

PfSET10 shows homology to histone lysine methyltransferases (HKMT) of the SET domain-containing protein family (Figure S3A) (Cui et al., 2008; Dillon et al., 2005). The SET domain distinguishes substrates and performs the catalytic activity (Trievel et al., 2002). Site-specific histone methylation serves either transcriptional gene activation or silencing and is dynamically regulated by interplay of HKMTs and histone lysine demethylases. PfSET10 is conserved within the Apicomplexan lineage; however, multiple sequence alignment based on structural information of PfSET10 revealed no similarity of the PfSET10 SET domain to any other known HKMT SET domain (Figure S3A). As well as a SET domain, PfSET10 contains a plant-like homeodomain (PHD) (Figure S3B), a structural motif involved in recognition of methylated or unmodified histone H3 (Mellor, 2006). The PHD finger domain displays highest similarity to uncharacterized PHD finger domains of *Arabidopsis thaliana* and *Toxoplasma gondii* (Figure S3B). Interestingly, PfSET10 PHD finger domain shares a number of amino acid residues with PHD finger domains that preferentially bind nonmethylated histone H3 (Figure S3B) (Lan et al., 2007; Org et al., 2008).

To determine the potential histone-binding properties of the PfSET10 PHD finger domain, we expressed and purified the PfSET10 SET/PHD (SET/PHD) domains including a control, which lacked the PHD finger (SET10/ΔPHD), using cell-free wheat germ expression (Figures 3A and S3C) (Tsuboi et al., 2010). In Far Western analysis using *P. falciparum* histone extracts as well as a histone peptide-binding assay, recombinant SET/PHD domain (Figures 3B and 3C) preferentially binds to histone H3 with highest affinity for the nonmethylated and monomethylated lysine 4 residue, which decreased upon di- and trimethylation or methylation of H3K9 (Figures 3B and 3C). Since the SET domain alone does not exert similar binding activity, the PHD finger was sufficient for H3 tail binding. PfSET10 binding to histone H2A cannot be excluded since histone H3 and H2A comigrate in the gel (Figure 3B) and PfSET10 colocalizes in IFA with histone H2A, but not H2.AZ (Figure 3D).

To determine if PfSET10 is a HKMT, we tested methyltransferase activity of the recombinant SET/PHD protein and a mutant form of the SET domain (ΔSET/PHD) (Figure S3C) using histone peptides, nucleosomes, or recombinant histones as substrates. The recombinant SET/PHD protein showed no detectable methyltransferase activity (data not shown). This observation indicated that PfSET10 may only be active as full-length protein or when present in a multiprotein complex (Briggs et al., 2001; Krogan et al., 2002; Nagy et al., 2002; Roguev et al., 2001). Therefore PfSET10 was immunoprecipitated from the 3D7SET10-HA parasites. Using recombinant histones as substrates, we were able to detect specific histone H3 methylation (Figure 3E) at an approximately 5-fold higher level compared to control (HA precipitate from 3D7), while methyltransferase activity toward histone H4 was considerably lower. Methylated lysines were detected with specific antibodies, and this indicated that incubation with PfSET10 increased di- and trimethylation of H3K4 (Figure 3E). PfSET10 also colocalized with the H3K4me1, me2, and me3 marks in the perinuclear region, which is also marked by H3K9ac (Figure 3F). While we cannot eliminate the possibility that enzymatic activity resides in another protein in the PfSET10 complex, sequence homology to the HKMT of the SET domain-containing protein family strongly indicate that PfSET10 preferentially targets and methylates lysine 4 (K4) on H3.

PfSET10 Interacts with PfActin-1

To identify proteins that are associated with PfSET10 in the *var* gene expression site, we performed immunoprecipitations on 3D7SET10-HA and 3D7 (negative control) using anti-HA antibody and identified associated proteins by LC-MS/MS (Figures 4, S4A, S4B, and Table S2). While a number of nuclear proteins were identified, including histone H2A (Figures 3B, 3D, and 4A), the presence of PfActin-1 was of interest, as it can be involved in gene transcription control through chromatin remodeling and directing movements of gene loci toward a target region (Visa and Percipalle, 2010). While PfActin-1 was distributed in speckles throughout the nucleus (Figure S4C), it showed colocalization with PfSET10 during late ring/early trophozoite stages (Figures 4B and S4C). The association of PfSET10 and PfActin-1 was confirmed by immunoprecipitation and immunoblot analysis (Figure 4B). These results indicate that PfActin-1 is a component of the *var* gene expression site.

C-Terminal GFP Tagging Compromises PfSET10 Function

To determine PfSET10 function in vivo and its potential involvement in *var* gene regulation, we attempted to disrupt the gene via homologous recombination (Figure S5A) and in parallel via a recently established conditional knockout system by which the expression of candidate proteins can be controlled (Figures S5B and S5C) (Armstrong and Goldberg, 2007). Interestingly, we were not able to disrupt the *pfset10* locus or destabilize PfSET10 expression. This indicated that PfSET10 was refractory to deletion, suggesting it is essential for parasite survival.

Initially, the endogenous *Pf*SET10 protein had been fused to GFP for live cell imaging analysis (Volz et al., 2010); however,

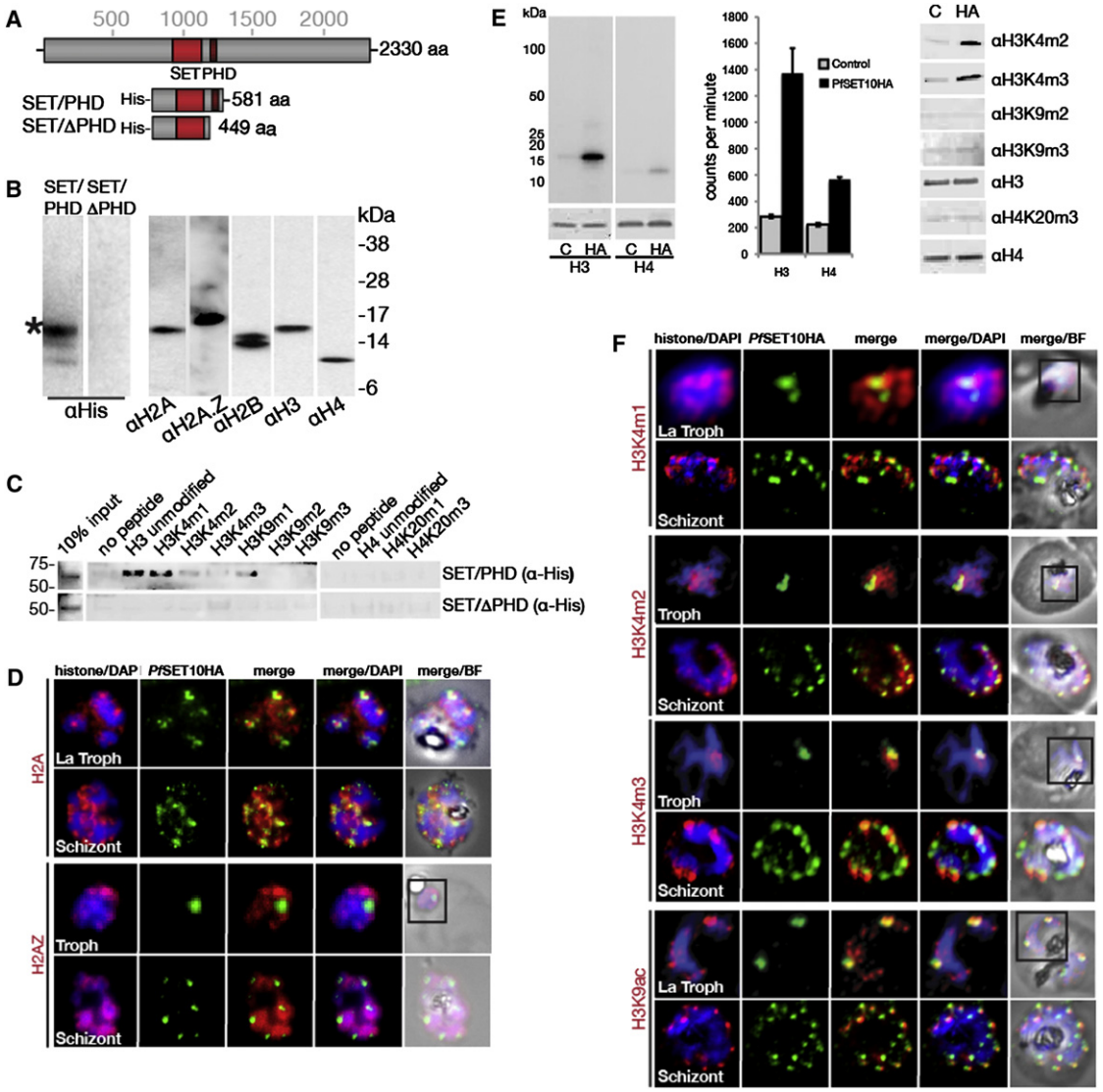

Figure 3. PfSET10 Is a Histone 3 Lysine 4 Methyltransferase

(A) Structure of PfSET10 showing the SET and PHD finger domains. Below are SET/PHD and SET/ΔPHD domains expressed as recombinant proteins with His tags.

(B) Far Western blot of *P. falciparum* histone extract using SET/PHD recombinant protein to show binding to histone H3 or potentially H2A (detected by anti-His antibodies; first panel). Subsequent panels are immunoblots of input material detecting H2A, H2A.Z, H2B, H3, and H4 with specific antibodies.

(C) Binding of SET/PHD to histone H3 and modified histone 3 peptides. SET/ΔPHD is shown as a negative control.

(D) Colocalization of histone H2A (two upper panels), but not H2A.Z (two lower panels) with PfSET10. The histones (red) were detected using specific antibodies and colocalized with PfSET10 (green) using anti-HA antibodies. Nucleus position is shown by DAPI.

(E) Immunoprecipitated PfSET10-HA has H3 methyltransferase activity in vitro. Recombinant human H3 and H4 were tested as substrates using radiolabeled SAM as methyl donor. The upper panels show the fluorograph in which H3 is preferentially labeled compared to H4. "C" is the immunoprecipitation from control parasites 3D7 while "HA" was from 3D7SET10HA. The bottom panels show a Coomassie-stained gel as a loading control. Data are represented as mean ± SEM of eight and six independent experiments of the activation assay using H3 and H4 as substrates, respectively. Reactions were also measured in a scintillation counter (middle panel). Methylated histone was detected using specific antibodies for the indicated histone marks (right panel).

(F) Colocalization of histone H3 marks with PfSET10. Shown are from top to bottom: H3K4m1, H3K4m2, H3K4m3, and H3K9ac. The histone marks (red) were detected using specific antibodies and colocalized with *Pf*SET10-HA (green) using anti-HA antibodies. Nucleus position is shown by DAPI staining. See also Figure S3.

we observed in at least two 3D7SET10-GFP parasite clones a growth phenotype compared to 3D7 and 3D7SET10-HA, suggesting the GFP tag had affected its function (Figure S5D). Since PfSET10 appeared refractory to genetic knockout or knockdown approaches, the phenotypic analysis of 3D7SET10-GFP parasites presented an opportunity to shed light on PfSET10 function. Localization studies implied that PfSET10 had a role in *var* gene regulation, so in order to determine potentially altered *var*

A 3D7/*Pf*SET10HA pulldown

PlasmoDB Accession[a]	Annotation	Coverage (%)	MW kDa	Unique Peptides
PF10_0068	RNA binding protein, putative	17.1	29.5	7
PFL1170w	Polyadenylate-binding protein, putative	4	97.2	5
PF10_0325	haloacid dehalogenase-like hydrolase, putative	10.8	32.8	4
MAL13P1.257.	Putative uncharacterized protein	28	18.7	4
PF13_0328	Profilerating cell nuclear antigen	12.4	30.6	4
PFF0940c	Cell division cycle protein 48 homologue, putative	7	92.4	4
PFL2215w	Actin-1	16	41.9	3
PF14_0655	RNA helicase-1	5.78	45.3	2
PF10_0063	DNA/RNA-binding (Alba protein), putative	22	119.8	2
PF11_0062	Histone H2B	21	13.1	2
PFL2060c	RabGDI protein	6.54	52.3	2
PFE1050w	Adenosylhomocysteinase	5.1	53.8	2
PFF0860c	Histone H2A	22	14.1	2

[a]Accession numbers (www.plasmodb.org) of nuclear proteins exclusively detected in the HA-bound fraction.

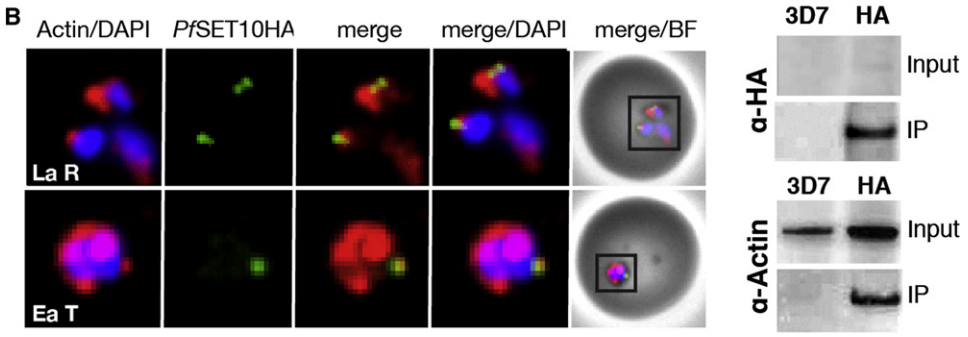

Figure 4. Identification of Proteins that Localize to the Active *var* Gene Expression Site

(A) Shown are proteins uniquely identified in the 3D7SET10-HA pull-down along with the number of unique peptides obtained and the percent of coverage. The PlasmoDB accession numbers of the genes identified are shown along with their functional annotation.

(B) IFA of PfSET10 (green) and PfActin-1 (red) with the nucleus (blue) stained with DAPI. First row, La R, late ring, second row, Ea T, early trophozoite. The right panels show immunoprecipitation of 3D7SET10-HA with anti-HA antibodies and detection of PfSET10-HA with anti-HA antibodies (left) and detection of PfActin-1 with PfActin-1 antibodies (right). See also Figure S4.

gene expression, 3D7SET10-GFP, 3D7, and 3D7SET10-HA parasites were selected for adherence to CSA to obtain those expressing *var2csa*. No changes in transcription of *var* genes other than *var2csa* was observed, indicating mutually exclusive expression in 3D7SET10-GFP remained intact (Figure 5A). However, we consistently observed an increased level of *var2csa* transcripts in 3D7SET10-GFP compared to 3D7 and 3D7SET10-HA (Figure 5A and data not shown). Increased *var2csa* transcription was further confirmed through the intraerythrocytic life cycle in 3D7SET10-GFP (Figure 5B). At ring stage (6–18 hr post invasion [p.i.]), we observed increased *var2csa* transcript in 3D7SET10-GFP, confirming the previous results. Strikingly, higher *var2csa* transcript levels were also observed at later stages (32–48 hr p.i.) in 3D7SET10-GFP parasites, while in 3D7 *var2csa* transcription was maintained in the typically quiescent poised state (Figure 5B).

The *var2csa* gene transcription that we have observed in late stages of 3D7SET10-GFP parasites suggests that chromatin around the gene must be accessible to the transcription machinery, similarly to that observed during ring stage at the active *var* locus. We performed chromatin-immunoprecipitation (ChIP) to determine the distribution of histone marks H3K4me2, H3K4me3, and H3K9ac, which are known marks for gene activation (Cui and Miao, 2010). In our control (3D7 parental parasites), which had been selected for *var2csa* expression, the level of these histone marks in the promoter region of the *var2CSA* gene was comparable to that observed for a euchromatic gene (*gbp130*) and higher than that of a silent *var* gene (PFL1960w) in ring and schizont stage parasites, respectively (Figure S6A). Moreover, H3K4me2 and me3 in the promoter of *var2csa* were enriched in 3D7SET10-GFP compared to the control (Figures 5C and S6B), with H3K9ac levels similar in both lines (Figure 5D). Therefore, the methylation levels of H3K4 of the poised *var* promoter are perturbed in 3D7/PfSET10GFP parasites, and this may account for the increased *var* gene expression pattern.

DISCUSSION

In *P. falciparum*, the nucleus is compartmentalized into heterochromatic and euchromatic regions, which distribution dynamically changes throughout the intraerythrocytic life cycle (Weiner et al., 2011). An intriguing feature in the *P. falciparum* nucleus is the presence of a zone of relaxed euchromatin within the nuclear

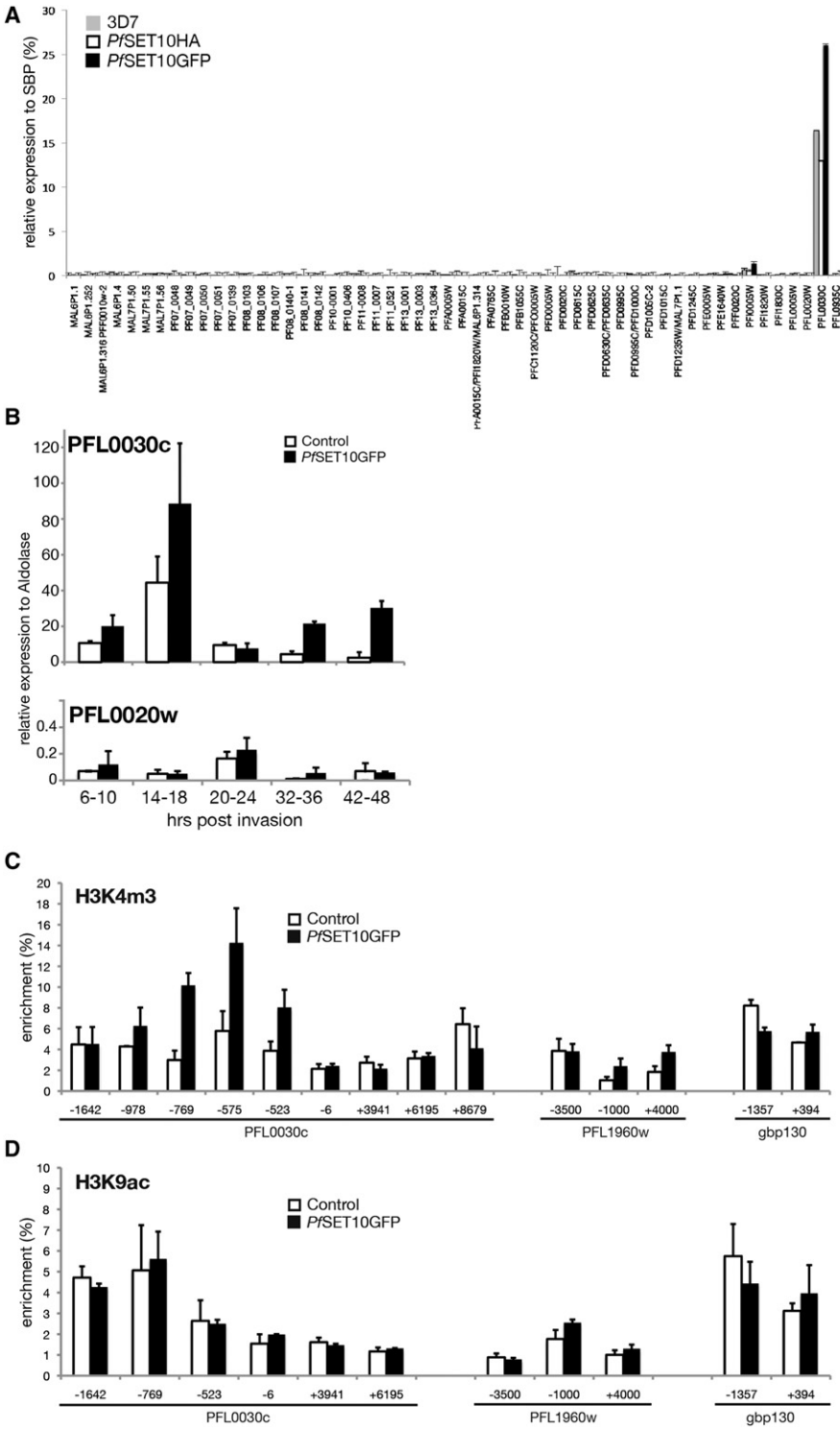

Figure 5. *Var2csa* Gene (PFL0030c) Is Dysregulated in 3D7SET10-GFP

(A) Quantitation of *var* gene transcripts for *var* in 3D7-CS, 3D7SET10-HA/CS, and 3D7SET10-GFP/CS parasites. Data are represented as mean ± SEM of two independent experiments.

(B) Quantitation of *var2csa* (PFL0030c) transcripts through the 48 hr cycle of 3D7-CS (control) versus 3D7SET10-GFP/CS compared to a silent *var* gene (PFL0020w). Data are represented as mean ± SEM of three independent experiments.

(C and D) (C) ChIP detecting the H3K4me3 mark in the *var2csa* (PFL0030c) gene compared to a silent *var* gene (PFL1960w) and *gbp130* gene as control. (D) ChIP detecting the H3K9ac mark as for (C). 3D7-CS served as wild-type control. Primer positions along the loci are indicated in relation to the start codon (ATG). See also Figures S5 and S6. Data in (C)are represented as mean ± SEM of two independent experiments and in (D) of three independent experiments.

periphery (Ralph et al., 2005). It has been speculated that this site may be specifically associated with transcription of the selected *var* gene from the 60 members of this multicopy virulence gene family (Duraisingh et al., 2005; Ralph et al., 2005; Voss et al., 2006). To date, this ''expression site'' was ill-defined and the molecular details of the mechanisms responsible for *var* gene translocation, activation, and poising remain almost completely unknown. In this study we have identified a protein, which we called PfSET10, that exclusively resides in this perinuclear expression site within an apparent euchromatic region. In contrast, we also confirmed that an inactive *var* gene was located within a chromosome end cluster marked by the presence of PfHP1 at the nuclear periphery. PfHP1 is involved in virulence gene silencing by maintaining a heterochromatic environment and establishing boundaries through its binding to histone mark H3K9me3 (Flueck et al., 2009). The active or poised *var2csa* gene did not colocalize with PfHP1 or the histone mark H3K9me3.

It is known from studies in eukaryotes, that transcriptionally active genes associate with so-called transcription factories (Osborne et al., 2004), which contain components of the transcription machinery such as RNA polymerase (Brown et al., 1999; Brown et al., 1997; Schübeler et al., 2000; Wang et al., 2004). Although human derived HeLa cells contain thousands of such transcription factories, numbers are restricted and several genes are translocated to the same site for transcription (Jackson et al., 1993). It is intriguing to speculate that the *var* gene transcription site, in which PfSET10 resides, might represent such a transcription factory to which *var* genes are translocated for activation.

As for *var* genes, members of the *rifin*, *stevor*, and *Pfmc-2TM* multigene families of *P. falciparum* undergo switched expression, indicating a potential role in antigenic variation (Kyes et al., 1999; Lavazec et al., 2007; Niang et al., 2009). Interestingly, it has recently been demonstrated, that a transcriptionally active episomal *rifin* promoter colocalizes with an active *var* promoter (Howitt et al., 2009). This indicates that members of the *rifin* multigene family may also translocate to this subnuclear expression site similar to the active *var*, and that possibly the expression site marked by PfSET10 is exclusively required for the transcription of virulence genes. At this stage, large-scale investigations into *P.falciparum* nuclear architecture, gene regulation, and movement are needed to clarify which set of genes translocate to the *var* gene/ PfSET10 transcription site for activation.

Interestingly, we identified PfActin-1 as a potential PfSET10-interacting protein. Nuclear actin can be part of the chromatin remodeling complex, involved in long-range chromatin organization and associated with the transcription machinery (Visa and Percipalle, 2010) or alternatively, together with myosin, may provide a molecular motor that steers gene loci toward the transcription site (Chuang et al., 2006; Dundr et al., 2007).

We have shown that PfSET10 has methyltransferase activity toward histone H3 lysine 4. In *P. falciparum*, three other SET-domain proteins have been predicted to have H3K4 methyltransferase activity, PfSET1 (PFF1440w), PfSET4 (PFI0485c), and PfSET6 (PF13_0293) (Cui et al., 2008). Localization studies in *P. falciparum* revealed that PfSET4 was present throughout the nucleus, while PfSET6 appeared to localize to the nucleolus (Volz et al., 2010). This observation suggests that *P. falciparum* H3K4 methylation may be performed in specific nuclear subcompartments by different HKMTs. Interestingly, in *Saccharomyces cerevisiae*, SET1 is the only enzyme responsible for H3K4 methylation (Briggs et al., 2001; Roguev et al., 2001), while in mammals at least ten known or predicted H3K4 methyltransferases exist. Deletion and phenotype analysis revealed that methyltransferases of the MLL family are not redundant in function; in contrast, they are specialized and differentially expressed being recruited to different gene loci (Glaser et al., 2006; Lee et al., 2006; Yu et al., 1995).

In various organisms, di- and trimethylation of the H3K4 site are enriched at actively transcribed genes (Bernstein et al., 2002; Santos-Rosa et al., 2002). In *S. cerevisiae*, poised genes are largely dimethylated at H3K4 (Ng et al., 2003; Pokholok et al., 2005; Santos-Rosa et al., 2002), while in vertebrates dimethylated H3K4 correlates with trimethylated H3K4 in highly active genes (Bernstein et al., 2005; Schneider et al., 2004). Genome-wide analysis of intraerythrocytic *P. falciparum* stages revealed that H3K4me3 was predominant at the intergenic regions, increasing in trophozoite and schizont stage parasites, in which transcription peaks (Bártfai et al., 2010).

In *P. falciparum*, the H3K4 mark colocalizes almost perfectly with the histone variant H2A.Z and histone H3K9ac (Bártfai et al., 2010). Interestingly, the histone variant H2A.Z is absent from subtelomeric and chromosome internal heterochromatic islands, in which *var* genes reside, and is complemented by H2A occupancy (Bártfai et al., 2010). Importantly in this context, transcriptional activation of a *var* gene requires a histone variant exchange at its promoter, during which H2A is replaced by H2A.Z (Petter et al., 2011). With the initiation of the S phase-dependent silencing of *var* genes, H2A.Z is lost from the promoter (Petter et al., 2011) possibly through deposition of newly synthesized canonical histones (Groth et al., 2007). While histones can be already largely acetylated prior to nucleosome assembly (Corpet and Almouzni, 2009), they generally lack methylation marks (Loyola et al., 2006). In our study, we observed that H3K4me3 levels, besides H3K4me2 and H3K9ac levels, are similarly high on an active compared to a ''poised'' *var* promoter, which is marked for reactivation in the next generation. This is consistent with the hypothesis that PfSET10 is the HKMT responsible for H3K4 methylation of the newly assembled nucleosome at the *var* promoter following replication, thereby maintaining a poised *var* promoter state. The recruitment of PfSET10 to its target site may be determined through DNA-specific binding factors, components of the transcription machinery or RNA (Ruthenburg et al., 2007), and with its PHD finger binding the unmethylated form of lysine 4 of a freshly deposited histone H3.

Previous studies have demonstrated that proteins specific for *var* gene regulation can be either amenable or refractory to genetic deletion (Duraisingh et al., 2005; Flueck et al., 2009; Tonkin et al., 2009). PfSET10 appears to be essential for parasite survival, since we were unable to obtain parasites in which PfSET10 has been disrupted. PfSET10 marks a transcription site and appears responsible for H3K4 methylation. Although it is not known at this stage how many genes utilize this site for transcription, PfSET10 removal would likely cause parasite lethality if expression of essential proteins is affected.

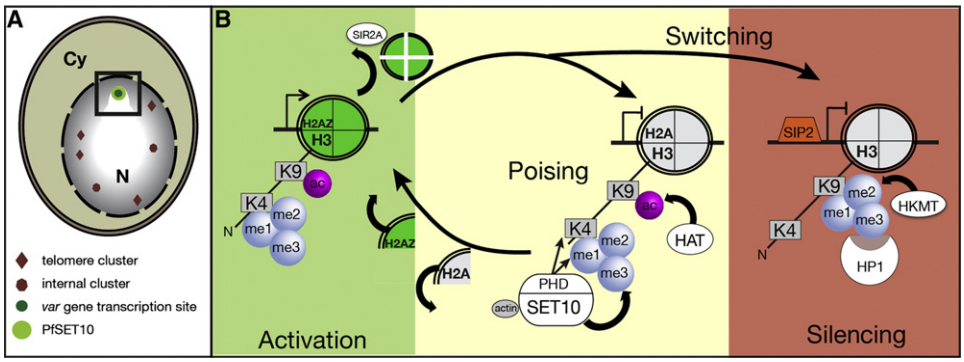

Figure 6. A Proposed Model for the Role of PfSET10 in Regulation of the Active *var* Gene in the Expression Site at the Nuclear Periphery

(A) PfSET10 localizes to the subnuclear region into which *var* genes are translocated for expression. Cy, cytoplasm in light brown; N, nucleus; the white area represents the euchromatic central part and subnuclear *var* gene expression site, while the gray area represents the heterochromatic nuclear periphery. A black square highlights the subnuclear expression site in which PfSET10 resides.

(B) Schematic presentation of histone H3 epigenetic modifications involved in *var* gene regulation and the proposed role of PfSET10. *Var* gene transcription associates with an open chromatin structure characterized by H3K4 trimethylation, H3K9 acetylation, and histone variant H2AZ. S phase dependent silencing of *var* genes is consistent with loss of histone variant H2AZ from the active *var* promoter. After replication, canonical histones such as histone H2A and H3 are incorporated to the *var* promoter providing a window of opportunity for switching and silencing. For poising, PfSET10 binds to non-or monomethylated H3K4, where it most likely sets the di- and trimethyl mark. This epigenetic profile is maintained during division and likely enables activation in daughter cells. Histone H2.AZ is deposited at the *var* promoter during ring stage. *Var* gene silencing involves movement of the *var* gene locus out of the expression site, deacetylation likely mediated by SIR2 homologs and H3K9 trimethylation by a yet unknown HKMT. PfSIP2 and PfHP1 are required for formation of stable heterochromatin. The color code used represents *green* for activation, *yellow* for poising and *red* for silencing.

Alternatively, absence of PfSET10 may lead to alterations in local chromatin structure, which in turn might affect chromosome organization and function. Already, the tagging of GFP to the endogenous *pfset10* locus led to changes in chromatin structure at the active and poised *var* gene locus and to altered levels of H3K4 methylation. Since the localization of the PfSET10-GFP is comparable to that of the HA-tagged protein, this increased H3K4 methylation could be the result of increased activity of the tagged PfSET10 and might account for the altered *var* gene transcription pattern. Alternatively, the GFP-tag of PfSET10 could interfere with the binding of a yet unknown transcriptional repressor, leading to a higher level of *var* transcription and consequently higher level of H3K4 methylation.

Modifications of core histones are involved in chromatin remodeling and gene regulation. Methylation of histones is thought to be important for heritable transmission of epigenetic information (memory) through cell division (Muramoto et al., 2010). PfSET10 is a *P. falciparum* histone 3 lysine 4 methyltransferase that defines the transcriptional zone into which a *var* gene is translocated for activation (Figure 6). The ''active'' *var* gene is activated in early ring stages and the resulting transcript translated into PfEMP1, followed by translocation to the infected erythrocyte membrane surface. This *var* gene is maintained in a poised state during cell division, ready for activation in the daughter cells of the next *P. falciparum* cycle. PfSET10 is likely required for the maintenance of the transcriptionally permissive chromatin environment of the active *var* promoter through division, by methylating the freshly incorporated histone H3, and involved in memory for the heritable transmission of epigenetic information during cell division. Our study has identified PfSET10 as a component of the *var* gene expression site and identifies it as an important regulator of virulence and phenotypic variation in *P. falciparum*, an important infectious agent of humans.

EXPERIMENTAL PROCEDURES

See a detailed version in Supplemental Information.

Parasite Culture and Transfection

PCR amplification was performed on *P. falciparum* strain 3D7 genomic DNA to obtain *Pfset10* 3′, which was cloned into a hemagglutinin (HA) vector, containing the human dihydrofolate reductase (*hdhfr*) gene (Crabb and Cowman, 1996; Fidock and Wellems, 1997), or into the GFP-FKBP-fusion or HA-FKBP-fusion vector, respectively, for conditional knockout construct generation (Armstrong and Goldberg, 2007; Fidock and Wellems, 1997). For gene deletion, PCR amplification was performed to obtain *Pfset10* 5′ and *Pfset10* 3′ fragments, which were cloned to the pCC4 vector (Maier et al., 2008). Primers are listed in Table S1.

P. falciparum 3D7 parasites were cultured and transfected as described (Crabb and Cowman, 1996; Armstrong and Goldberg, 2007). Integration of the 3′ gene replacement constructs was by homologous recombination (Figures S1 and S5). 3D7SET10-HA and 3D7 samples were analyzed by western blot, and PFSET10-HA was detected with anti-HA antibody.

Recombinant Protein Expression

The PfSET10 fragment containing SET and PHD finger domains was codon-optimized for wheat and synthesized (Epoch Biolabs, Inc.) and expressed in a wheat germ cell-free expression system (Cell-Free Sciences) (Tsuboi et al., 2010). The in vitro histone peptide-binding assay was performed with a Biotinylated Protein-Protein Pull-Down Kit (Pierce).

Far Western

P. falciparum histones were run on a 4%–12% Bis-Tris gel, and the membrane was incubated with 2 μg/ml of recombinant protein and anti-His and secondary antibody, then detected using Amersham ECL Western Blotting Detection Reagent (GE Healthcare).

HMKT Assay

To purify HA-tagged, full-length PfSET10 protein from 3D7SET10-HA and 3D7 parasites, 2×10^9 late-stage parasites were harvested, and cell extracts were prepared. HA-tagged PfSET10 was purified by anti-HA beads (Sigma). The in vitro histone methyltransferase assay was performed as described (Fingerman et al., 2008).

Nuclear Fractionation

Nuclear and cytoplasmic protein fractions from 3D7SET10-HA parasites were obtained as described (Voss et al., 2002), analyzed by western blot, and *Pf*SET10-HA was detected with anti-HA antibody.

Fluorescence Microscopy, Combined IFA/FISH and Transmission Electron Microscopy

Methanol-fixed cells were analyzed using mouse anti-HA 3F10 (Roche), anti-H3K4m1 (Abcam, ab8895), anti-H3K4m2 (Abcam, ab7766), anti-H3K4m3 (upstate, 05-745), anti-H3K9ac (upstate, 06-942), anti-H3K9m3 (Abcam, ab8898), anti-histone H2A (abcam, ab18255), anti-PfH2.AZ (Petter et al., 2011), anti-PfHP1 (Petter et al., 2011), anti-PfActin-1 (Riglar et al., 2011), and Alexa Fluor 488 conjugated anti-mouse IgG (Molecular Probes). IFA/FISH was carried out as described (Flueck et al., 2009). Statistical analysis was performed using a two-sided, two-sample Kolmogorov-Smirnov test. Immunoelectron microscopy was performed on 3D7SET10-HA schizonts, which were fixed (4% formaldehyde, 0.1% glutaraldehyde), dehydrated, and embedded in LR Gold resin (Electron Microscopy Sciences, Fort Washington, PA).

Chromatin Immunoprecipitation (ChIP) and Transcriptional Profiling

ChIP analysis was performed as described (Flueck et al., 2009). Primers are listed in Table S1. RNA was harvested by cell lysis in TRIzol (Invitrogen), purified as described (Kyes et al., 2000), and cDNA was generated using Superscript III Reverse Transcriptase (Invitrogen). Quantitative RT-PCR was performed as described (Duffy et al., 2009; Petter et al., 2011). Primers are listed in Table S1.

Protein Pull-Down

HA-tagged, full-length *Pf*SET10 protein and interacting proteins from 3D7/*Pf*SET10HA and control line 3D7 were purified using anti-HA beads (Sigma). Proteins were separated on a SDS-PAGE and stained in colloidal Coomassie. Protein bands were excised and submitted for LC-MS/MS analysis. After separation of the pull-down fraction in SDS-PAGE, membranes were probed with either anti-HA 3F10 (Roche) or anti-PfActin-1 (Riglar et al., 2011).

CSA Binding Assay

Enrichment of parasites expressing *var2csa* PfEMP1 or PFL0020w PfEMP1 through subsequent CSA- or ICAM-binding assays was performed as described (Duraisingh et al., 2005).

SUPPLEMENTAL INFORMATION

Supplemental Information includes six figures, two tables, Supplemental Experimental Procedures, and Supplemental References and can be found online with this article at doi:10.1016/j.chom.2011.11.011.

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