PfSETvs methylation of histone H3K36 represses virulence genes in Plasmodium falciparum

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The variant antigen Plasmodium falciparum erythrocyte membrane protein 1 (PfEMP1), which is expressed on the surface of P. falciparum-infected red blood cells, is a critical virulence factor for malaria1. Each parasite has 60 antigenically distinct var genes that code for a different PfEMP1 protein. During infection the clonal parasite population expresses only one gene at a time before switching to the expression of a new variant antigen as an immune evasion mechanism to avoid the host antibody response2–5. The mechanism by which 59 of the 60 var genes are silenced remains largely unknown6–7. Here we show that knocking out the P. falciparum variant-silencing SET gene (here termed PfSETvs), which encodes an orthologue of Drosophila melanogaster ASH1 and controls histone H3 lysine 36 trimethylation (H3K36me3) on var genes, results in the transcription of virtually all var genes in the single parasite nuclei and their expression as proteins on the surface of individual infected red blood cells. PfSETvs-dependent H3K36me3 is present along the entire gene body, including the transcription start site, to silence var genes. With low occupancy of PfSETvs at both the transcription start site of var genes and the intronic promoter, expression of var genes coincides with transcription of their corresponding antisense long noncoding RNA. These results uncover a previously unknown role of PfSETvs-dependent H3K36me3 in silencing var genes in P. falciparum that might provide a general mechanism by which orthologues of PfSETvs repress expression in other eukaryotes. PfSETvs knockout parasites expressing all PfEMP1 proteins may also be applied to the development of a malaria vaccine.

In addition to histone deacetylases (HDACs)8,9, histone lysine methyltransferases (HKMTs) or histone lysine demethylases (HKDMs) may have critical roles in controlling gene expression in P. falciparum10–11. There are a total of ten predicted P. falciparum HKMTs (PfHKMTs) belonging to the SET domain superfamily, two PfHKDMs of the LSD1 family and three PfHKDMs of the Jumonji-related family10,12 (Supplementary Table 1). However, the key factor for var gene silencing remains unknown.

We therefore examined whether PfHKMTs or PfHKDMs are key factors in controlling mutually exclusive expression of the var gene family by attempting to knock out all of the PfHKMT (PfSET) genes and three of the PfHKDM genes in a P. falciparum clone, 3D7 (Fig. 1a and Supplementary Fig. 1). Four out of nine PfSET genes and all three studied PfHKDM genes could be genetically disrupted (Fig. 1b and Supplementary Fig. 1), suggesting that the other five PfSET genes are essential for the parasite in the asexual blood stage. Gene expression microarray analyses showed that the knockout (Fig. 1c, d and Supplementary Fig. 1c) of the gene previously referred to as PfSET2 (ref. 10) (PlasmoDB gene ID: PF3D7_1322100) led to the expression of virtually all var genes in the ring stage (Fig. 1e and Supplementary Table 2). By contrast, knockout of any other PfSET or PfHKDM genes did not alter the transcription of the var gene family in 3D7 (Supplementary Fig. 1e–j and Supplementary Tables 3–8). In addition, some members of other clonally variant gene families (rifin and stever) plus the var gene family account for most of the genes upregulated in the P. falciparum 3D7 lacking the SET2 gene (3D7SET2Δ) (Supplementary Fig. 2 and Supplementary Table 2). Therefore, we renamed this P. falciparum variant-silencing SET gene PfSETvs. Activation of the majority of var genes by SETVsΔ was further corroborated by quantitative PCR (qPCR) at 18 h after invasion in both 3D7 (Fig. 1f) and another P. falciparum clone, Dd2 (Supplementary Fig. 3), indicating that PfSETvs is involved in broadly silencing var genes.

To determine whether PfSETvsΔ activated multiple var genes in a single infected red blood cell (iRBC), we tested whether different types of var genes could be transcribed in a single 3D7SETVsΔ iRBC by RNA fluorescence in situ hybridization (FISH). Each combined RNA FISH of two representative var transcripts indicated co-expression of all three types of var genes in an individual 3D7SETVsΔ nucleus (Fig. 2a). The tested var transcripts colocalized with each other at a particular site of the nuclear periphery (Fig. 2a). Transcription of a control gene, seryl-tRNA synthetase (PF3D7_0717700), did not occur at this site (Fig. 2a), suggesting that var genes have a specific transcriptionally active site, in agreement with previous findings13. Moreover, our results showed that multiple var transcripts also colocalized at the single peripheral site of 3D7SETVsΔ nuclei, even though the genomic loci of these var genes were diverse (Supplementary Fig. 4a–c). Taken together, our results demonstrate multiple var transcripts in one nucleus and suggest that a var-specific nuclear compartment exists for active transcription of multiple var genes.

To determine whether parasites transcribing multiple var genes are able to translate and transport multiple PfEMP1 proteins to the surface of iRBCs, a live-cell immunofluorescence assay (IFA) was performed with rat and rabbit antibodies to different PfEMP1 proteins. As expected, the gelatin-enriched parasite presented knobs on the surface of iRBCs in both 3D7 and 3D7SETVsΔ (Fig. 2b, c). Furthermore, surface expression of multiple PfEMP1 proteins on a single 3D7SETVsΔ iRBC was observed by confocal microscopy (Fig. 2d and Supplementary Fig. 4d). It is

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**Figure 1** | Knockout of *PfSETvs* leads to expression of all *var* genes.

a, Schematic diagram of the *PfSETvs* gene knockout strategy by using plasmid pHTK. 3′ F, 3′ flanking fragment for crossover recombination; 5′ F, 5′ flanking fragment for crossover recombination; E, EcoRV; hDHFR, human dihydrofolate reductase; P, DNA probe for Southern blot analysis; TK, thymidine kinase; SET, SET domain. b, Summary of knockout studies for nine *PFHKTm* and three *PFHKDMs* (*PFJlSD1*, *PFJlmc1* and *PFJlmc2*) genes. KO, knockout; no, failed to knockout gene; yes, succeeded in gene knockout. c, d, Southern blot analysis using a DNA probe (P) from downstream of the knocked out SET domain of the *PfSETvs* gene (see also panel a) for *PfSETvs*Δ in 3D7 (c) and Dd2 (d). The sizes of three different hybridization bands from the integrated (In) or wild-type (WT) genomes and the episomal plasmid (EP) are indicated to the right. bp, base pairs. e, Comparative transcriptome analysis of wild-type 3D7 and 3D7*SETvsΔ* at 18 h after invasion. x axis (wild-type 3D7) and y axis (3D7*SETvsΔ*) are logarithmic and correspond to relative signal of hybridization to each gene shown as a dot (see also Supplementary Table 2). All *var* genes with authentic hybridization signals are shown in red. The dominantly expressed *var* gene (PF3D7_1240600) in wild-type 3D7 is indicated by a red arrow. f, qPCR analyses of transcriptional upregulation (log2 ratio of *PfSETvs*Δ to wild-type parasites) of *var* genes in 3D7*SETvsΔ* at 18 h after invasion. Type of *var* gene (A, B, C or E) is shown at the top. The dominantly expressed *var* gene and a second gene expressed at a low frequency in the wild-type 3D7 population are indicated by red arrowheads. Experiments were repeated three times. Error bars represent s.e.m.

**Figure 2** | Simultaneous expression of multiple *var* genes in single 3D7*SETvsΔ* iRBCs. a, Two-colour RNA FISH (top) and statistical analyses of colocalization (bottom) of each two types of *var* transcripts in 3D7*SETvsΔ* by using gene-specific probes (Supplementary Fig. 4a). Seryl-tRNA synthetase (Ser) transcript served as a negative control. Average numbers of counted nuclei are listed under each tested group, n = 3. Error bars represent s.e.m. P values were obtained using a one-tailed Student’s t-test. **P < 0.01. b, c, Electron microscope of gelatin-selected 3D7 and 3D7*SETvsΔ* iRBCs.

Typical knobs in scanning electron microscopy (b) and transmission electron microscopy (c) pictures are indicated by red arrowheads. d, Live-cell IFA using rat and rabbit antisera to various *PIEMP1* proteins to detect co-expression of different *PIEMP1* proteins on the surface of 3D7*SETvsΔ* iRBCs. Wild-type 3D7 iRBCs is shown to the right. No staining is seen. DAPI (4′,6-diamidino-2-phenylindole, blue) is used to mark the parasite nucleus. Types of *var* genes are shown in parentheses. Scale bars, 1 μm (a, b), 0.5 μm (c) and 1.5 μm (d).
was observed only in the telomeric and subtelomeric heterochromatin regions of the 14 P. falciparum chromosomes plus several discrete genomic regions where all of the var genes are located at either 18 or 42 h after invasion. However, compared with other histone lysine methylations, H3K36me3 was greatly reduced in the entire gene body of var genes in 3D7 ΔSETvs at 18 h after invasion (Fig. 3d and Supplementary Figs 8 and 9), indicating a direct positive correlation of H3K36me3 with PfSETvs activity. Considering the extremely low level of H3K36me2 at var loci in wild-type 3D7 (Supplementary Fig. 7a), only H3K36me3 is functionally important for var gene regulation. PfSETvs may di- and trimethylate H3K36, as these markers were also reduced at the transcription start site (TSSs) of activated PfSETvs may di- and trimethylate H3K36, as these markers were also reduced at the transcription start site (TSSs) of activated var genes owing to PfSETvs (Supplementary Fig. 7c–g). Interestingly, similarly high levels of H3K36me3 were observed in both wild-type 3D7 and 3D7ΔSETvs at 42 h after invasion when var genes were silent (Fig. 3e), indicating at least one other PHKMT that catalyses H3K36me3 in P. falciparum schizont IRBCs. In addition, our data showed that none of the var transcripts colocalized with H3K36me3 in the nuclei (Supplementary Fig. 10). Collectively, our data suggest that the PfSETvs-dependent H3K36me3 is specifically involved in var gene silencing.

Notably, H3K36me3 was also observed for a high enrichment at the 3′ end of 400 ring-stage-active genes (other than var, rifin and stevor genes) compared to 400 ring-stage-silent genes (see gene lists in Supplementary Table 9) in both wild-type 3D7 and 3D7ΔSETvsΔ (Fig. 3f, g), indicating that PfSETvs-independent H3K36me3 may contribute to transcriptional elongation, as reported in other eukaryotes15–17, and might compensate for the global levels of H3K36me3 in 3D7ΔSETvsΔ (Supplementary Fig. 6c, d). We next examined whether the reduction of H3K36me3 by PfSETvsΔ is specifically associated with activation of parasite clonally variant genes. Among 5,276 P. falciparum genes, 59 out of 59 var genes, 97 out of 150 rifin genes (including 69 A- and 28 B-type rifin genes) and 18 out of 29 stevor genes belonged to the top 250 genes with highest reduction of H3K36me3 by PfSETvsΔ (Fig. 3h). Furthermore, the same gene group is enriched for increased expression as determined by microarray experiments (Supplementary Table 10). Our data indicate that H3K36me3, controlled by PfSETvs, has a repressive role in silencing parasite clonally variant gene families.

To corroborate further the role of H3K36me3 in var gene silencing, we examined histone modification at the TSS of an active var gene (PF3D7_1240600) and a silent var gene (PF3D7_1200600) in the wild-type 3D7, both of which are active in 3D7ΔSETvsΔ (Fig. 4a, b). Because of

Figure 3 | PfSETvs-dependent H3K36me3 is specifically associated with var gene silencing. a, Integrative genomic view of ChIP-seq analysis of H3K36me3 along 3D7 (black) and 3D7ΔSETvsΔ (red) chromosomes at 18 h after invasion. Sixty var genes distributed along P. falciparum chromosomes 1–13 are indicated by solid (forward orientation) and open (reverse orientation) arrows. Chromosomal numbers are shown to the left. Regions are boxed for a detailed view represented in b and c. A scale bar representing 200 kilobases (kb) is shown to the right of chromosome 1. b, c, At 18 h and 42 h after invasion, integrative genomic view of H3K36me3 distributed at the 5′ end of chromosome 4 representing a region that includes the telomere, subtelomere, type A and B var genes (b), and at the middle of chromosome 7 representing a type C var gene cluster (c) in 3D7(black) and 3D7ΔSETvsΔ (red).

f, g, Distribution of H3K36me3 across the gene bodies of 400 ring-stage-active genes (red) and 400 ring-stage-silent genes (blue) (see gene list in Supplementary Table 9) in wild-type 3D7 (f) and 3D7ΔSETvsΔ (g). Each gene was equally divided into 20 bins. Total reads of each bin by ChIP-seq were normalized by total uniquely mapped reads. h, Statistical analysis of the correlation between reduction of H3K36me3 and upregulation of var, rifin and stevor gene families. 5,276 parasite genes were sorted from low to high levels of H3K36me3 in 3D7ΔSETvsΔ normalized by that in 3D7. Expression fold change of each gene by PfSETvsΔ was shown on the top panel (see also Supplementary Table 10). Distribution of all of var (red), rifin, including A- and B-type rifin genes (green) and stevor (blue) genes is shown along the parasite genes (gold). In the top 250 H3K36me3-reduced genes boxed by dash lines, numbers of var (red), rifin (green), stevor (blue) and other genes (grey) compared to their total numbers were shown in a pie chart at the bottom. Hypergeometric test was computed for the var (P = 3.4×10⁻⁶⁰), rifin (P = 9.7×10⁻⁵⁰) and stevor (P = 1.73×10⁻¹⁷) gene families to gauge their significance of upregulation in the reduction of H3K36me3.
high sequence similarity in the 5’-untranslated region, including the TSS and the intronic promoter of var genes, ChIP-qPCR but not ChIP-seq can be used in these regions (Fig. 3b, c). In wild-type 3D7, the TSS occupancy of H3K36me3 is considerably higher in the silent var gene compared to the active one (Fig. 4a, b). By contrast, the two var genes studied both exhibited low levels of H3K36me3 at the TSS in 3D7/ΔSETvsΔ, consistent with their active expression (Fig. 4a, b). H3K9me3, a transcriptional silent mark, showed similar profiles as H3K36me3 (Fig. 4a, b), whereas two active marks, H3K4me3 and H4 acetylation, were present at the TSSs of active genes in both wild-type 3D7 and 3D7/ΔSETvsΔ (Fig. 4a, b). In addition, the similar results were observed in three other var genes representing type A (PF3D7_0400400), type B (PF3D7_0300100) and type C (PF3D7/_0617400) (Supplementary Fig. 1). Altogether, our data support the idea that the high level of H3K36me3 at the TSS region is involved in transcriptional repression.

It is worth noting that each var gene harbours an intronic promoter driving the transcription of an antisense long non-coding RNA (lncRNA) of unknown function14. Our ChIP-seq data showed that two active var genes (PF3D7_1240600 and PF3D7_0900100) in wild-type 3D7 populations (Fig. 1f) had low levels of H3K36me3 at the 3’ end of exon 1, whereas silent var genes had high levels of H3K36me3 at the same region (Supplementary Fig. 12), suggesting a positive correlation between PISETVs-dependent H3K36me3 occupancy and var lncRNA silencing. To explore this concept further, histone modification profiles in the 3’ portion of var exon 1a as a proxy for the lncRNA promoter was examined by ChIP-qPCR, as the introns of var genes are highly conserved among the gene family. Our results showed similar trends of H3K36me3 between the TSSs of var genes and their corresponding 3’ but not 5’ portions of exon 1 (Fig. 4a, b and Supplementary Fig. 11), consistent with the observation by strand-specific qPCR that active transcription of var genes coincides with the expression of the corresponding antisense lncRNAs at 8–18 h after invasion (Fig. 4c, d and Supplementary Fig. 13). These results demonstrated a correlated upregulation of var genes and their corresponding lncRNAs in association with low occupancy of the PISETVs-dependent H3K36me3 at the TSS.

To investigate further the biological function of PISETVs in var gene silencing, a triple haemagglutinin (HA) tag was fused in frame to the carboxy terminus of PISETVs in 3D7/SETvsΔHA (Supplementary Fig. 14a–d). The resulting PISETVs–HA protein, like wild-type PISETVs, still contributed to the mutually exclusive expression of the var gene family (Supplementary Fig. 14e). Furthermore, IFA analysis showed that PISETVs–HA located at multiple nuclear sites, one of which colocalized with H3K36me3 in 3D7/SETvsΔHA (Supplementary Fig. 14f), suggesting that the enzymatic activity of PISETVs for H3K36me3 might require additional factors at the single perinuclear site. ChIP-qPCR results showed that, at 18 h after invasion, PISETVs–HA was not enriched at the TSS and in the intronic promoter region of the active var gene (Fig. 4e), and instead tended to increase at these regions of silent var genes tested in 3D7/SETvsΔHA (Fig. 4f and Supplementary Fig. 14g–i). No comparable enrichment of PISETVs–HA was observed in a var-unrelated silent gene (PF3D7_0424100) (Supplementary Fig. 14j). Taken together, our data indicate that PISETVs–HA specifically localizes to the TSSs and intronic promoters for var gene silencing, in association with the PISETVs-dependent H3K36me3 (Fig. 4g).

In this study we have shown that the H3K36 methylation system is differentiated into at least two distinct forms in P. falciparum, with the PISETVs-dependent system functioning in a negative regulatory capacity (Fig. 4g), and the second independently of it alongside the elongating RNAPII (Supplementary Fig. 14k). Cognates of the PISETVs-dependent mechanism for gene silencing might also exist in other eukaryotes in the cases of previously reported members of the ASH1-like subclade, such as Caenorhabditis elegans MES-4 (ref. 19) and D. melanogaster ASH1 (ref. 20), and perhaps explain the association between H3K36me3 and silent genes in zebrasfish sparrow21 and the pericentromeric heterochromatin in mouse embryonic stem cells and fibroblasts22. In the RNAPII-related mechanism, H3K36me3 generated by the SETD2 subclade enzymes recruits HDACs15 and prevents incorporation of acetylated histones23 in transcribed gene bodies to prevent cryptic transcription initiation inside active genes. Given the role of lncRNAs as scaffolds

**Figure 4** | PISETVs and H3K36me3 repress var gene expression at the TSS. a, b, ChIP-qPCR of the active 3D7 var gene PF3D7_1240600 (a) and a silent 3D7 var gene PF3D7_1200600 (b) with antibodies to H3K36me3, H3K9me3, H3K4me3 and histone H4/K5/K8/K12/K16 acetylation in both 3D7 and 3D7/ΔSETvsΔ at 18 h after invasion by using three different PCR primer sets schematized in Supplementary Fig. 7b. 3ex1, 3’ end of exon 1; 5ex1, 5’ end of exon 1. c, d, Expression profiles of messenger RNA and antisense lncRNA transcribed from PF3D7_1240600 (c) or PF3D7_1200600 (d) at five different time points after invasion as shown in the figures. Expression levels of var transcripts were normalized to expression of a housekeeping gene, arginyl-tRNA synthetase (PF3D7_0913900). The forward and reverse primers of the 3ex1 PCR primer set (Supplementary Fig. 7b) were used for antisense lncRNA and mRNA reverse transcription, respectively. Type of var gene and its transcription status are shown in parentheses. Experiments were repeated three times. Error bars represent s.e.m. e, f, ChIP-qPCR of the active 3D7 var gene PF3D7_1240600 (e) and a silent 3D7 var gene PF3D7_1200600 (f) with a mouse antibody to HA in 3D7/SETvs–ΔHA at 18 h after invasion by using the same PCR primers in a, g. Summary diagram showing that the PISETVs-dependent H3K36me3 enriched along the entire gene body of silent var genes, including the TSS of var genes and the respective intronic antisense promoter, leads to silencing of both var mRNA and antisense lncRNA. Ac, acetylation.
recruiting Set2 histone methyltransferase and Set3 histone deacetylase complex to repress transcription initiation in yeast24,25, it would be interesting to investigate whether the antisense lncRNA might regulate var gene expression in a similar manner25.

The factor that activates individual var genes in the wild-type parasite still remains unknown. It may be a mechanism that randomly turns on var genes at a low rate. We previously found that only 1 in 200 parasites expresses the reticulocyte binding protein-like homologue 4 (Rh4) ligand in Dd2 (ref. 26), controlled by H3K9me3 (ref. 27), and a similar mechanism involving PISETvs may exist for var genes. Recent work demonstrates that PIEMP1 proteins are key targets of humoral immunity26. However, malaria immunity is acquired only slowly after years of repeated exposure that, in part, reflects the time required for an individual to experience a sufficient number of variant antigens. The SETvsΔ parasite could be used as an antimalarial vaccine because of its ability to express all PIEMP1 proteins, to which the antibody would provide efficient protective immunity against malaria.

**METHODS SUMMARY**

Gene knockout in *P. falciparum* clones 3D7 and Dd2 was carried out using the double-crossover recombination strategy. After PCR screening, the positive knock-out parasites were cloned and confirmed by Southern blot analyses. Transcriptome changes in each 3D7 knockout clone were initially analysed by the PFSANGER Affymetrix array at indicated time points after invasion. Transcriptional upregulation of most of var genes in either 3D7SETvsΔ or Dd2SETvsΔ at 18 h after invasion were further corroborated by qPCR. To evaluate the co-expression of multiple var genes in individual iRBCs, two-colour RNA FISH using different var antisense lncRNAs was performed at 18 h after invasion of 3D7SETvsΔ. Our phylogenetic analysis (Supplementary Fig. 5) strongly suggested PISETvs as a H3K36 methyltransferase. We therefore investigated the distribution changes at global level of H3K36me2/3 in 3D7 caused by PISETvs by ChIP-seq assay. As controls, we tested other histone methylations (H3K4me3, H3K9me3 and H4K20me3) in parallel. In addition, histone modification changes at the TSS region of var genes were investigated by ChIP-qPCR. To explore the biological function of PISETvs in regulating var gene silencing further, a triple HA tag was fused in frame to the C terminus of PISETvs in 3D7SETvsΔ by allelic exchange as described previously21. For strand-specific qPCR with reverse transcription assay, transcription of antisense lncRNAs driven by the var intronic promoter was investigated at five indicated time points after invasion of 3D7SETvsΔ or wild-type 3D7. DNA primers used in this study are listed in Supplementary Table 11.

Full Methods and any associated references are available in the online version of the paper.

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METHODS
Parasite culture and transfection. P. falciparum clones 3D7 (initially isolated from the Netherlands) and Dd2 (initially isolated from Vietnam) were cultured in human O− erythrocytes according to standard procedures. For gene deletion, PCR amplification was performed on P. falciparum strain 3D7 genomic DNA to obtain gene-specific 5' and 3' flanking fragments, which were cloned into Spe I/BglII (5')- and EcoR I/Ncol (3')-digested pHTK vector. Names of the twelve targeted genes (Fig. 1d) and PCR primers are listed in Supplementary Table 11. Transfection and knockout selection were performed as described previously. In brief, 250 µl of packed IRBCs (5−10% ring parasites) were transfected by electroporation with 100 µg of the transfection pHTK plasmid. Positive (WR99210, 2 nM) and negative (ganciclovir, 20 µM) drug selection were applied for selecting a population of parasites in which the plasmid-derived human DHFR gene (for WR99210 selection) had been integrated via double crossover homologous recombination into the endogenous targeted gene locus, and the episomal plasmid carrying the Herpes simplex virus 2 TK gene (for ganciclovir self-killing selection). Selected knockout parasites were further confirmed by PCR screening (See also Supplementary Fig. 1a) before being cloned by limiting dilution.

Southern blotting. Southern blot analyses on PSETv3A or PSETv3HA parasites were performed using the DIG High Prime DNA Labelling and Detection Starter Kit (Roche) according to the product manual. In brief, genomic DNA was digested by EcoRV for 4 h at 37 °C for 4 h at 37 °C and summarization of the probe sets. For qPCR analysis, RNA was isolated and purified as described above. First, strand complementary DNA was synthesized by either random primer mixes or commercial antibody to the secondary horseradish peroxidase-conjugated goat anti-rabbit IgG (Sigma), using the rabbit antisera to the PfSETvs peptide and detected by an enhanced chemiluminescence (ECL) kit (Thermo Scientific). Total proteins from wild-type 3D7 were analysed as a control. Anti-PfSETvs peptide, diluted at a ratio of 1:500, and the secondary horseradish peroxidase-conjugated goat anti-rabbit IgG (Sigma), diluted 1:10,000, were incubated with western blot polyvinylidene difluoride (PVDF) membrane for ECL development. To determine reaction specificity of a commercial antibody to P. falciparum H3K36me3 (Cell Signalling), 1 µg of each four synthesized peptides with the P. falciparum-specific histone H3K36 sequence (PH3K36, biotin−GKKPKHRYPRG; PH3K36m1, biotin−GIK(me)KPKHRYPRG; PH3K36m2, biotin−GK(m2)KPKHRYPRG; PH3K36m3, biotin−GK(m3)KPKHRYPRG) was dotted on the PVDF membrane for western blot analysis as described above. To detect the effect of PSETv3A on histone lysine methylations, total parasite proteins from wild-type 3D7 and Dd2 PSETv3A extracted at 18 h after invasion were carried out for western blot analysis using rabbit antibodies to H3K36me3 (Cell Signalling), H3K36me2 (Abcam), H3K4me3 (Abcam) and H3K9me3 (Millipore), respectively. Antibody to histone H3 (Millipore) was used as a control. Rabbit anti-HA (Abcam) was used to detect PSETv3s-HA in 3D7SEv3sHA. The western blot analysis was performed as mentioned above.

Microarray analyses. To analyse global gene expression profiles in the asexual stage, RNA from wild-type 3D7 and Dd2 PSETv3A were extracted from highly synchronized parasite cultures at 18 h (ring), 30 h (trophozoite) and 42 h (schizont) after invasion by using TRIzol (Life Technologies) according to the product manual and further digested with RNase free DNase (Ambion) to remove the RNA contamination. RNA hybridization was performed using the PFSANGER Affymetrix array at the microarray facility of the National Cancer Institute. In brief, 10 µg of total RNA was reverse-transcribed and biotin-labelled. Hybridizations were carried out at 45 °C for 16 h with constant rotation at 60 g. Gene arrays were then scanned at an emission wavelength of 570 nm at 1.56 µm pixel resolution using a confocal scanner (Affymetrix GeneChip Scanner 3000 7G). After scanning, the hybridization intensity for each probe was measured using Affymetrix GCOS version 1.3 software. The raw data was then transferred to our in-house software for background adjustment, normalization and summarization of the probe sets.

qPCR. For qPCR analysis, RNA was isolated and purified as described above. First, strand complementary DNA was synthesized by either random primer mixes or gene-specific primers using Superscript III Reverse Transcriptase (Life Technologies) according to product manual. PCR primers used for detecting mRNA expression of 3D7 var genes were as described previously. Primers for detecting transcripts from each Dd2 var gene and for 3D7 var lncRNAs were designed in this study (Supplementary Table 11). qPCR was performed on a Q5 Multi-colour Real-time PCR Detection System (Bio-Rad) with a program of 1 cycle of 5 min at 95 °C, 40 cycles of 30 s at 95 °C, 30 s at 50 °C and 30 s at 60 °C. A housekeeping gene, arginyl- tRNA synthetase (PF3D7_0913900), was used to normalize the transcriptional level of each var gene.

Live-cell-infected RBC IFA. Live-cell IFA for infected RBCs was performed as described previously with minor modifications. In brief, IRBCs were washed in 1% BSA in PBS (BSA/PBS) and the pellet was re-suspended in 200 µl BSA/PBS. Antibodies specific for various PIEM1 proteins listed in Supplementary Table 3b were used at a 1:50 dilution and incubated at room temperature (23 °C) for 30 min. After washing three times in BSA/PBS, cells were fixed with 2.5% paraformaldehyde and 0.01% glutaraldehyde for 10 min at room temperature and washed with BSA/PBS. Subsequently, cells were incubated with Alexa-488-conjugated goat anti-rabbit IgG (Life Technologies) and Alexa-594-conjugated goat anti-rat IgG (Life Technologies) for 30 min at room temperature and washed with BSA/PBS containing 0.1% Triton X-100 and mounted with prolong gold DAPI. Images were captured on a Leica SP2 confocal microscope and visualized using Bitplane Imaris software.

Scanning and transmission electron microscopy. Scanning and transmission electron microscopy were performed as described previously with modifications. For scanning electron microscopy (SEM), IRBCs were gently allowed to settle on silicon chips for 20 min at room temperature in an 8-well chamber slide (Labtek). Freshly prepared fixative (2.5% glutaraldehyde, 3% paraformaldehyde, 0.05 M phosphate buffer, 4% sucrose) was added to the cells and incubated at room temperature for 1 h. All subsequent processing was carried out in a Polco Biowave laboratory instrument; a balanced mechanical microfluidic system (Ted Pella) at 250 W and 20 in Hg (mercury) vacuum. The chips were post-fixed with 1% osmium tetroxide−0.8% potassium ferricyanide in 0.1 M sodium cacodylate, followed by rinsing with distilled water and dehydration in a graded ethanol series. The specimen was critical point dried in a Bal-Tec CPD 030 drier (Bal-Tec AG) and coated with 80 A of iridium using an IBS ion beam sputter (South Bay Technology). SEM samples were imaged using a Hitachi S/8000 SEM (Hitachi High Technologies). For transmission electron microscopy, parasites were fixed with 2.5% glutaraldehyde, 3% paraformaldehyde, 0.05 M phosphate buffer and 4% sucrose at room temperature for 2 h. The cells were post-fixed in a microwave with 1% osmium tetroxide−0.8% potassium ferricyanide in 0.1 M sodium cacodylate, followed by 1% tannic acid in distilled water, and stained en bloc with 1% aqueous uranyl acetate. They were then rinsed with distilled water and dehydrated in a graded ethanol series. The pellets were then infiltrated and embedded in Spurr’s resin which was polymerized overnight in a 68 °C oven. Thin sections (90 nm) were cut using a UC6 ultramicrotome (Leica Microsystems) and stained with 4% aqueous uranyl acetate and Reynolds’s lead citrate before viewing on a 120 kV Tecnai Biotwin Spirit TEM (FEI). Digital images were acquired with a Hamamatsu XR-100 digital camera system.

FISH. Synchronized ring-stage parasites were released from IRBCs by 0.15% saponin treatment followed by fixation with 4% paraformaldehyde in 1 × PBS overnight at 4 °C. The fixed parasites were washed twice with 1 × PBS, then deposited on a microscope slide (Fisher Scientific) as a monolayer and subjected to RNA FISH in the conditions as described previously. For combined immunofluorescence and RNA FISH, parasites were fixed in 4% paraformaldehyde in 1 × PBS and re-suspended in 2 × saline-sodium citrate buffer and fixed again in 4% paraformaldehyde for 15 min before IFA for detection of H3K36me3 by using the antibody to H3K36me3 (Cell Signalling) with 1:100 dilutions. For the individual var genes of the RNA FISH probes, DNA templates were amplified by PCR from 3D7 genomic DNA with primers shown in Supplementary Table 11. For the template of the exon 2 probe for the var gene family, the exon 2 regions were amplified with types A and C primers as described previously. The products were pooled for labelling. The PCR products were purified by Gel Extraction kit (Qiagen) and used in probe preparation with a Biotin- or Fluorescein-High Prime kit (Roche). Images were captured by using a Nikon Eclipse 80i microscope with a CoolSnap HQ2 camera (Photometrics). Primers used in amplification of individual var probes are described in Supplementary Table 11.

ChiP-seq and ChiP-qPCR. Highly synchronized cultures of ring-, trophozoite- and schizont-stage parasites were prepared for the ChiP study. Crosslinked chromatin was prepared by adding 1% formaldehyde to the culture for 5 min followed by addition of glycine to 0.125 M final concentration. After sonication lysis, nuclei were isolated by homogenization in 10 mM Tris at pH 8.0, 3.0 mM MgCl2 and 0.2% Nonidet P-40, and collected on a 0.25 M sucrose−buffer cushion and suspended in

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SDS buffer (1% SDS, 50 mM Tris, pH 8.0, 10 mM EDTA, protease inhibitors). Chromatin was sheared by sonication in a Bioruptor UCD-200 (Diagenode) for 10 min at 30-s intervals, power setting high, to a size of 300–800 bp. Chromatin samples were frozen and stored at −80 °C. ChIP was performed as described previously. In brief, commercially available antibodies to H3K36me3 (Cell Signaling), H3K4me3 (Abcam), H3K9me3 (Millipore), H3K20me3 (Abcam) and histone H4K5/K8/K12/K16 acetylation (Abcam) were added to crosslinked samples of wild-type 3D7 and 3D7
SETvs
D
, or a mouse anti-HA (Abcam) to 3D7
SETvsHA
samples, and incubated at 4 °C, followed by the addition of 10 μl A/G beads and further incubation for 2 h. After washing with buffers containing 100, 150 and 250 mM NaCl, immuno-precipitated DNA was eluted and purified using PCR purification columns (Qiagen). The resulting double-stranded DNA was then end repaired, followed by adding an A base at the ends. Illumina paired-end index adaptor was ligated and size selected. A 16-cycle PCR was then carried out with Phusion Hot Start High-Fidelity DNA Polymerase (Finnzymes) to generate the final ChIP-seq library. We used Illumina HiSeq 2000 to perform the single-end sequencing (50 cycles). Quality sequencing reads were mapped against the组装 psychotic range across euukaryotes were collected using the Position-Specific Iterative Basic Local Alignment Search Tool (PSI-BLAST) program. The SET domains and the associated AWS domains were aligned using the MUSCLE program. The tree was constructed using two methods: (1) a preliminary tree was obtained using the approximately-maximum-likelihood method implemented in the FastTree 2.1 program under default parameters. This gave an idea of the positions of key members; and (2) a complete tree was constructed using the MEGA 5.1 program with the following parameters: four distinct gamma distributed rate categories and one invariant were used for modelling amongst site variation, the WAG matrix with frequencies, was used as the substitution model; the maximum-likelihood searched used the close neighbour exchange method. The tree was bootstrapped using 10,000 resampling of estimated log-likelihood bootstrap percentage resamplings with the MOLPHY package. The tests for alternative topology were carried out using the CONSEL program for the Shimodaira–Hasegawa test and these overwhelmingly rejected the grouping of the apicomplexan clade with either the NSD subclade (P < 10−5) or the SETD2 subclade (P < 10−7).