Revisiting the initial steps of sexual development in the malaria parasite *Plasmodium falciparum*

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Human to vector transmission of malaria requires that some blood-stage parasites abandon asexual growth and convert into non-replicating sexual forms called gametocytes. The initial steps of gametocytogenesis remain largely uncharacterized. Here, we study this part of the malaria life cycle in *Plasmodium falciparum* using PfAP2-G, the master regulator of sexual conversion, as a marker of commitment. We demonstrate the existence of PfAP2-G-positive sexually committed parasite stages that precede the previously known committed schizont stage. We also found that sexual conversion can occur by two different routes: the previously described route in which PfAP2-G-expressing parasites complete a replicative cycle as committed forms before converting into gametocytes upon re-invasion, or a direct route with conversion within the same cycle as initial PfAP2-G expression. The latter route is linked to early PfAP2-G expression in ring stages. Reanalysis of published single-cell RNA-sequencing (RNA-seq) data confirmed the presence of both routes. Consistent with these results, using plaque assays we observed that, in contrast to the prevailing model, many schizonts produced mixed plaques containing both asexual parasites and gametocytes. Altogether, our results reveal unexpected features of the initial steps of sexual development and extend the current view of this part of the malaria life cycle.

The transcription factor AP2-G was recently identified as the master regulator of sexual conversion in malaria parasites10,11. This protein, which belongs to the ApiAP2 family of DNA-binding proteins12, is essential for gametocyte production in *P. falciparum*, as it regulates the transcriptional program that mediates sexual conversion13,14. The *pfap2-g* gene is the only member of the ApiAP2 family in *P. falciparum* that carries the heterochromatin marks of epigenetic silencing H3K9me3 and PIHP1 (refs 15,16), and it belongs to a subset of genes that show clonally variant expression17,18. Depletion of factors involved in heterochromatin formation results in activation of the gene and enhanced rates of sexual conversion19,20. Altogether, these observations indicate that sexual conversion is regulated at the epigenetic level: the *pfap2-g* locus is silenced by H3K9me3-based heterochromatin in asexually growing parasites, and transition to a permissive chromatin state, which requires the protein GDV1 (refs 19,21), leads to activation of the gene and subsequent sexual conversion19. Once PfAP2-G is expressed it locks in commitment by driving its own transcription11.

PfAP2-G provides a much needed specific marker for the highly unexplored sexually committed parasite stages. Here we used PfAP2-G to characterize the initial steps of sexual differentiation in *P. falciparum* and re-examined the idea that an additional round of replication after commitment is an obligate step.

**Results**

When PfAP2-G is stabilized at the early ring stage, sexual conversion can occur within the same cycle of stabilization. In the E5-PfAP2-G-DD parasite line19 (E5-HA-DD hereafter), in which endogenous PfAP2-G is fused to the FKBP destabilization domain and a haemagglutinin (HA) tag, PfAP2-G is stable only in the...
Fig. 1 | Gametocytes can form at the same cycle of PIAP2-G activation. 

a, Proposed nomenclature for the initial steps and stages of sexual differentiation. Sexual conversion is marked by the onset of gametocyte-specific expression of proteins absent from any replicating blood stages (asexual or sexually committed). Sexual commitment is defined as a cell state that deterministically results in sexual conversion at a later point. Currently, sexually committed forms are morphologically indistinguishable from their asexual counterparts. Sexual rings, which have also been named as ring gametocytes or gametorings, are still morphologically indistinguishable from asexual rings, but they mature into stage I gametocytes. Expression of known protein markers for the different stages is shown. Vertical lines indicate re-invasion.

b, E5-HA-DD cultures with PIAP2-G stabilized at the ring stage (+Shld) form stage I gametocytes before re-invasion. Gametocytes were detected as mononucleated parasites positive for the early gametocyte marker Pfg27. Similar results were obtained in four independent experiments. Scale bar, 5 µm.

c, Gametocytes develop from E5-HA-DD cultures treated simultaneously with Shld and GlcNAc at the ring stage. Similar results were obtained in five independent experiments. Scale bar, 5 µm.

d, e, Sexual conversion (proportion of parasites that differentiate into gametocytes, see Methods) in E5-HA-DD cultures with Shld added at different hours post-invasion (h.p.i.). GlcNAc was added at the ring stage of the next cycle to determine total sexual conversion (d) or simultaneously with Shld to measure same cycle conversion (SCC) (e). Individual data points, mean and s.e.m. from three independent biological replicates (except for 10–15 h.p.i., n = 2) are shown. P values were calculated using one-way analysis of variance. f, FACS-sorted PIAP2-G–eYFP-negative schizonts of the E5-eYFP line produce gametocytes within the first cycle after re-invasion. Bar charts show the proportion of PIAP2-G–eYFP-positive schizonts in unsorted controls and sorted samples, and the proportion of Pfs16-positives among pigmented parasites (within 3 h of sorting and 48 h later), as determined by IFA. In each experiment, more than 1,000 parasites of each sample were counted. Data are presented as the average and s.e.m. of four independent experiments (except for Pfs16 ‘sorted’, n = 3). P values were calculated using a two-tailed unpaired t-test with equal variance. Scale bars, 5 µm.
presence of the Shield-1 (Shld) ligand, and in its absence PfAP2-G is degraded and gametocytes never form\(^1\). When adding Shld to synchronized E5-HA-DD cultures at the ring stage, we observed single-nucleated Pfg27-positive parasites within as little as ~30 h after Shld addition, when the majority of parasites were still at the schizont stage (Fig. 1b). As Pfg27 is a well-established marker of early gametocytes from stage I onwards\(^1,2,3,4,6,23\), this result suggests that asexual parasites can convert directly into gametocytes without going through an additional round of multiplication after PfAP2-G stabilization. To test this idea, we treated E5-HA-DD cultures at the ring stage simultaneously with Shld and N-acetyl-d-glucosamine (GlcNAc), which at the concentration used (50 mM) inhibits schizont maturation\(^3,4,6\). Indeed, mature gametocytes emerged in these cultures (Fig. 1c), demonstrating that gametocytes can form without going through the PfAP2-G-positive committed schizont stage. This suggests that sexual conversion can occur through a same cycle conversion (SCC) route, in addition to the canonical next cycle conversion (NCC) route involving an additional round of replication after commitment. We define the new SCC route as conversion to sexual gametocyte in the progeny of PfAP2-G-negative schizonts, without additional replication. This implies that commitment, as marked by PfAP2-G expression, and conversion to gametocyte can occur within the same cycle.

Next, we investigated whether the timing of PfAP2-G stabilization during the intraerythrocytic cycle affects the frequency of sexual conversion via the NCC or SCC routes. E5-HA-DD cultures were tightly synchronized to a 5 h window and Shld was added at 0–5, 10–15, 20–25 or 30–35 h post-invasion (h.p.i.). When we added GlcNAc at the ring stage (~5–10 h.p.i.) of the next cycle to measure sexual conversion by either route, we observed high conversion rates (≥25%) regardless of the timing of PfAP2-G stabilization (Fig. 1d). In sharp contrast, when we measured conversion within the same cycle of PfAP2-G stabilization by adding GlcNAc simultaneously with Shld, conversion was maximal when Shld was added shortly after invasion (0–5 h.p.i.) and nearly negligible when PfAP2-G was stabilized at 20–25 h.p.i. or later (Fig. 1e). These results indicate that when PfAP2-G is stabilized at the early ring stage, sexual conversion frequently occurs within the same growth cycle through the SCC route, whereas PfAP2-G stabilization at later stages results in conversion predominantly at the next cycle via the NCC route. Gametocyte activation and egress assays did not reveal differences between gametocytes generated by the SCC or the NCC routes (Supplementary Fig. 1).

PfAP2-G-negative schizonts produce gametocytes within the first cycle after re-invasion. The E5-HA-DD line shows an unusually high sexual conversion rate when Shld is added (Fig. 1d,e). In other parasite lines, high rates of pfap2-g activation would result in high sexual conversion and lower multiplication rates, posing a fitness cost, but this does not occur in the E5-HA-DD line maintained in the absence of Shld. This raises the possibility that the regulation of pfap2-g expression may be altered in this line. To determine whether parasites in which pfap2-g is regulated normally can convert via the SCC route, we tagged endogenous PfAP2-G with the fluorescent marker enhanced yellow fluorescent protein (eYFP) using the CRISPR–Cas9 system (E5-eYFP line, Supplementary Fig. 2) and used tightly synchronized E5-eYFP late schizont cultures to sort PfAP2-G–eYFP-negative parasites (Supplementary Fig. 3) by fluorescence-activated cell sorting (FACS). The almost complete absence of PfAP2-G–eYFP-negative parasites after sorting was validated by IFA (Fig. 1f). IFA analysis with antibodies against the very early gametocyte marker Pfs16 (refs. \(^1,2,3,4,6\)) conducted 48 h after sorting, when most parasites that continued asexual growth were at the schizont stage, revealed the presence of new Pfs16-positive gametocytes (Fig. 1f). For these experiments, we used Pfs16 instead of Pfg27 as a gametocyte maker because we found that it is an absolutely specific marker that is never expressed in PfAP2-G-deficient parasites and its expression starts earlier during gamocyte development (Supplementary Fig. 4), as previously reported\(^6\). These results show that gametocytes can arise from PfAP2-G-negative asexual schizonts in the first cycle after re-invasion, confirming conversion via the SCC route.

Schizonts can produce pure sexual or pure asexual plaques as well as mixed plaques. Previous reports described that in immobilized cultures individual schizonts produce plaques composed of either only gametocytes or only asexual forms\(^7\), which led to the generally accepted view that sexual commitment always occurs in the cycle before conversion\(^1,2,4,6.23\). In light of our identification of the SCC route we wanted to re-examine this observation: if commitment and conversion can occur within the same cycle, parasites arising from an asexual schizont could become gametocytes independently of the choice made by their sibling progeny, resulting in mixed plaques (Fig. 2a). One-cycle plaque assays (Fig. 2) performed with tightly synchronized cultures of the wild-type 3D7 subclone E5 indeed revealed that almost 40% of gametocyte-containing plaques consisted of a mixture of Pfs16-positive gametocytes and multinucleated asexual parasites (Fig. 2d). This proportion of mixed plaques cannot be explained by multiply-infected erythrocytes in the overlaid schizonts preparation (Supplementary Table 1 and Supplementary Fig. 5). To confirm that the levels of mixed plaques reflect the use of the SCC route, in these experiments we included E5-HA-DD cultures treated with Shld at times that result in conversion predominantly via the SCC or the NCC routes, which revealed a much higher proportion of mixed plaques in SCC cultures (Fig. 2c,d). Additional characterization of the plaques is provided in Supplementary Fig. 5b,c. Of note, IFA analysis of PfAP2-G in >200 PfAP2-G-positive mature schizonts of the E5-HA-DD and E5-HA lines\(^1,2,3,4,6\) (the latter expressing HA-tagged endogenous PfAP2-G) did not identify any schizont containing a mixture of HA-positive and HA-negative merozoites, supporting the view that mixed plaques arise from direct conversion via the SCC route and not from single schizonts containing sexual and asexual merozoites (Fig. 2b). Abundant mixed plaques were also observed in assays performed with a different 3D7 stock (Supplementary Table 2 and Supplementary Fig. 5d). Altogether, these results confirm that sexual conversion can occur at the same cycle of commitment in wild-type parasites.

Single-cell transcriptomics confirms same cycle sexual conversion. In a previous study, E5-HA-DD cultures with Shld added at the ring stage (~4–16 h.p.i.) were used for single-cell RNA-seq analysis either during the commitment cycle (~30–36 and ~42 h.p.i. of the same cycle of Shld treatment, cycle 1) or at the next cycle (stage I gametocytes, cycle 2)\(^1,3,4,6\). Cluster analysis of transcriptional patterns of individual parasites revealed that the majority of cycle 2 gametocytes fall within clusters 13 and 14. Notably, some parasites from the commitment cycle (cycle 1) also fall within these two clusters (Fig. 3a,b). Comparing the relative abundance of Shld-treated versus untreated cells from cycle 1 shows an especially strong enrichment of treated cells for cluster 13 (Fig. 3c and Supplementary Fig. 6). These results suggest that cycle 1 parasites that fall within cluster 13 (4.8% of the total cycle 1 cells, Fig. 3b) converted into stage I gametocytes at the same cycle of Shld treatment using the SCC route.

To confirm that parasites from the commitment cycle (cycle 1) that fall in cluster 13 are actually stage I gametocytes, we identified the most upregulated genes expressed by cycle 2 stage I gametocytes in cluster 13 as compared to expression in cells from other clusters. For all of these genes, which include known early gametocyte markers such as pfg27 and pfexp02 (refs. \(^2,5,6\)) and also for the well-established early gametocyte markers pfs16 and pfg14.748, transcript levels in cycle 1 cluster 13 cells were higher than in cycle 1 cells from other clusters (Fig. 3d,e, Supplementary Table 3 and Supplementary Figs. 6 and 7). However, average pfap2-g expression was similar
between cluster 13 and other clusters. This is because, in contrast to gametocyte-specific markers, pfap2-g is abundantly expressed in sexually committed cells\(^1\), which account for pfap2-g expression within other clusters (Fig. 3c and Supplementary Figs. 6 and 7).

In spite of the high overall transcriptional similarity between cycle 1 and cycle 2 cluster 13 cells (Fig. 3a,d), we identified a small number of transcriptional differences that are probably associated with the route of conversion (Supplementary Fig. 8). The early gametocyte marker pfpg14.748 (ref. 28) and two other genes with the route of conversion (Supplementary Fig. 8). The early gametocyte marker (ref. 28) and two other genes with the route of conversion (Supplementary Fig. 8).

**Expression of pfap2-g is maximal at the ring and mature schizont stages.** The discovery of the SCC route predicts that initial pfap2-g expression can be activated in rings. To test this idea, we employed the gametocyte non-producer F12 line, which contains a non-sense mutation that results in a non-functional PfAP2-G protein\(^10,30\). This makes it ideally suited to determine the temporal dynamics of pfap2-g transcription in the absence of gametocytes or the PfAP2-G transcriptional feedback loop\(^15,13\). Time-course analysis of pfap2-g relative transcript levels in F12 revealed a clear peak of expression in 10–15 h.p.i. rings. By contrast, in E5 cultures transcript levels were already high at 0–5 h.p.i. and decreased until 30–35 h.p.i. before increasing again at 40–45 h.p.i. (Fig. 4a).

In addition to late schizonts, 40–45 h.p.i. samples already contain early rings of the new growth cycle. To determine whether mature schizonts actually express pfap2-g, we collected RNA at 45–50 h.p.i. from cultures treated with the cyclic GMP-dependent protein kinase (PfPKG) inhibitor ML10, which blocks merozoite egress and re-invasion\(^11\). These experiments revealed expression of pfap2-g in mature schizonts (Fig. 4b), which was confirmed by analysis of magnet-purified schizonts (Supplementary Fig. 9a). As a cautionary note, we observed that measuring pfap2-g transcripts from RNA extracted with Trizol directly from Percoll-purified schizonts yielded artefactual results (Supplementary Fig. 9a).

To directly assess the effect of PfAP2-G protein on pfap2-g expression, we used cultures of the E5-HA-DD line with or without Shld. As expected, the pfap2-g temporal expression profile in E5-HA-DD without Shld was similar to F12, with peak expression at 10–15 h.p.i., whereas in Shld-treated cultures expression was high at all the time points analysed except at 30–35 h.p.i., similar to E5 (Fig. 4c). The higher expression in Shld-treated versus untreated cultures is consistent with PfAP2-G autoregulating the expression of its own gene\(^10,13\). Characterization of the expression of other early gametocyte markers\(^1\) in the same time-course conditions reveals that PfAP2-G autoregulation is the primary mechanism for the regulation of pfap2-g expression.

**Fig. 2 | Plaque assays reveal that some individual schizonts generate mixed sexual and asexual plaques.** a, Schematic of the possible types of plaque originated from schizonts expressing or not expressing PfAP2-G (expression indicated by red nuclei). Early gametocytes expressing the Pfs16 marker are indicated in green. Mixed plaques can arise from asexual schizonts if some rings activate PfAP2-G expression and convert within the same cycle (SCC route). b, Representative IFA images of pure asexual, mixed and pure sexual plaques. Asexual parasites are multinucleated schizonts, Pfs16-negative and contain a large hemozoin pigment, whereas gametocytes are mononucleated, Pfs16-positive and have small hemozoin pigment granules. Arrowheads indicate free hemozoin pigment (residual body) from the parental schizont that originated the plaque. Single-nucleated parasites without hemozoin pigment or Pfs16 signal are merozoites that failed to develop. Images are representative of four independent experiments, each including at least three different samples. Scale bars, 5 µm. c, Distribution of plaque types in the wild-type line E5, E5-HA-DD treated with Shld at 0–5 h.p.i. (favouring the SCC route) and E5-HA-DD treated at ~30 h.p.i. of the previous cycle (favouring the NCC route). At least 100 plaques of each culture were counted in each experiment. d, Distribution of pure and mixed plaques only among plaques containing ≥1 Pfs16-positive parasite. At least 100 plaques of each culture containing ≥1 sexual parasite were counted in each experiment. In c and d, values are the average of three independent biological replicates (red dots) and s.e.m. The distribution of plaque types in c and d was significantly different between E5, E5-HA-DD SCC and E5-HA-DD NCC conditions (P=0.000 using a two-tailed Fischer’s exact test).
Fig. 3 | Single-cell RNA-seq identification and characterization of SCC gametocytes. a, Cluster analysis of previously published single-cell transcriptomics data from parasites of the E5-HA-DD line treated with Shld at ~4–16 h.p.i. and isolated at ~30, ~36 or ~42 h.p.i. of the same cycle (cycle 1) or at ~42 h.p.i. of the next cycle (cycle 2, stage I gametocytes). See Supplementary Fig. 6 legend for a detailed definition of t-distributed stochastic neighbour embedding (t-SNE) axes. The bar chart on the right shows the proportion of parasites from cycle 1 and cycle 2 in selected clusters (normalized per number of cells in each sample) from an analysis of 7,472 cells (5,736 from cycle 1 and 1,736 from cycle 2). The cycle 1 versus cycle 2 compositions of cluster 13, cluster 14 and the combined remaining clusters were all non-randomly distributed (all $P < 1 \times 10^{-18}$, two-sided exact Poisson test). b, Distribution of cells from cycle 1 (Shld-treated and untreated) and cycle 2 between the different clusters from an analysis of 10,509 cells. Cycle 1 values are normalized to account for the number of cells analysed at each time point. c, Relative abundance of cycle 1 Shld-treated (bright shading) or untreated (pale shading) cells within each cluster. Abundance is normalized to account for the number of cells collected at each treatment condition. Numbers at the right show the relative enrichment (RE) for treated cells within each cluster and the number of cells in each cluster ($n$). Error bars indicate 95% confidence intervals based on the two-sided exact Poisson test. d, Average expression of cluster 13 cycle 2 gametocyte markers (Supplementary Table 3 and Supplementary Figs. 6 and 7) shown for Shld-treated cycle 1 cells outside cluster 13 (5,552 cells) or within cluster 13 (184 cells), and for Shld-treated cycle 2 cluster 13 cells (1,003 cells). Expression is normalized to 10,000 transcripts per cell. Mean and estimated 95% confidence intervals are shown. e, Same analysis as in d but for additional gametocyte markers.

experiments showed that pfg14.744 transcripts are absolutely specific for gametocytes, whereas pfgexp5 transcripts and low levels of pfs16 and pfg27 transcripts can be detected in the absence of functional PfAP2-G and gametocytes (Supplementary Fig. 10), consistent with previous reports.

Overall, these results show that basal pfap2-g transcript levels peak at the ring stage, suggesting that activation is possible at this stage and probably involves a ring-specific ApiAP2 transcription factor. By contrast, in the presence of functional PfAP2-G, transcript levels are high at all stages except for late trophozoites/early schizonts, consistent with previous reports. Our conclusions are largely independent of the gene used for normalization (Supplementary Fig. 9b). Analysis of pfap2-g transcript levels during gametocyte development revealed a progressive decrease after stage I (Supplementary Fig. 9c).

PfAP2-G expression and sexual commitment can start before the schizont stage. We previously described that PfAP2-G–HA is localized in the nucleus of some parasites in cultures synchronized to the ring, trophozoite or schizont stage. However, if sexually committed rings and trophozoites exist, in IFA experiments they would be morphologically indistinguishable from sexual rings and stage I gametocytes, respectively. Using Pfs16 protein as a gametocyte marker that is absent from sexually committed parasites, we found that PfAP2-G–HA is expressed in the nucleus from presumably committed rings and trophozoites exist, in IFA experiments they would be morphologically indistinguishable from sexual rings and stage I gametocytes, respectively. Using Pfs16 protein as a gametocyte marker that is absent from sexually committed parasites, we found that PfAP2-G–HA is expressed in the nucleus from presumably committed trophozoites to stage I gametocytes (Fig. 5a), although some stages could not be unambiguously identified by this approach (see below). The distribution of PfAP2-G within the nucleus shows very limited overlap with heterochromatin, as expected from the euchromatic location of the majority of its predicted targets, but...
in many parasites the PfAP2-G signal appeared to concentrate in the nuclear periphery (Supplementary Fig. 11a,b). At later stages of sexual development (stage II–V gametocytes) the PfAP2-G–HA signal is near background levels over the whole parasite and does not show nuclear localization (Fig. 5b). Western blot analysis confirmed a decrease of PfAP2-G–HA protein levels with gametocyte development (Supplementary Fig. 11c).

We observed Pfs16 signal in some parasites without detectable pigment (Fig. 5a, sexual ring), consistent with the observation that expression of this marker starts before the previously reported 30–40 h.p.i. (ref. 26 and Supplementary Fig. 4). These results suggest that single-nucleated, hemozoin-containing parasites that are not express Pfs16 at detectable levels yet. To confirm the occurrence of committed forms preceding the schizont stage, we analysed tightly synchronized E5-HA-DD cultures by IFA with Shld (45–50 h.p.i.) or Shld added at 0–5 h.p.i. Re-synchronization to a 5 h age window was performed between cycles 1 and 2. In cycle 1, pfap2-g expression was significantly higher in the presence of Shld compared to the −Shld condition at all time points analysed (P = 0.002, 0.002 and 0.027 at 10–15, 20–25 and 30–35 h.p.i., respectively, using a two-sided t-test with equal variance). In all panels, transcript levels were normalized against ubiquitin-conjugating enzyme (uCe). Values are the average of two (c) or three (a,b) independent biological replicates (red dots). Error bars in a and b are s.e.m.

The SCC route implies that de novo expression of PfAP2-G can start before nuclear replication. Indeed, we observed PfAP2-G–eYFP-positive rings among the progeny of FACS-sorted PfAP2-G–eYFP-negative schizonts of the E5-eYFP line analysed by IFA 20 h after sorting (Fig. 5e), indicating that PfAP2-G–HA is present in the nucleus of early but not older stage I gametocytes. The SCC route implies that de novo expression of PfAP2-G can start before nuclear replication. Indeed, we observed PfAP2-G–eYFP-positive rings among the progeny of FACS-sorted PfAP2-G–eYFP-negative schizonts of the E5-eYFP line analysed by IFA 20 h after sorting (Fig. 5e), indicating that PfAP2-G–HA is present in the nucleus of early but not older stage I gametocytes.
Fig. 5 | PfAP2-G expression dynamics. a, IFA analysis of PfAP2-G–HA and the early gametocyte marker Pfs16 in E5-HA cultures. Images are representative of six independent experiments. Scale bars, 2 µm. b, IFA analysis of PfAP2-G–HA and the gametocyte marker Pfpg27 in E5-HA GlcNAc-treated gametocyte cultures. Images are representative of three independent experiments. Scale bar, 2 µm. c, IFA analysis of PfAP2-G–HA and the gametocyte marker Pfs16 at different h.p.i. in Shld-treated E5-HA-DD cultures. d, Proportion of stage I gametocytes (Pfs16-positive) positive for PfAP2-G–HA nuclear signal. In c and d, values are the average of two independent biological replicates (red dots). e, IFA analysis of PfAP2-G–eYFP expression in rings arising from FACS-sorted eYFP-negative schizonts of the E5-eYFP line. IFA was performed 3 h (‘sorted’) and 20 h (‘sorted + 20h’, ≤20 h.p.i. rings) after sorting. Pfs16-expression and P values were determined as in Fig. 1f. Values are presented as the average and s.e.m. of four independent experiments (red dots). f, Sexual conversion in E5-HA-DD cultures treated with Shld at 20–25 h.p.i. and with GlcNAc at 5–10 h.p.i. of the following cycle. Shld was removed at the times indicated by the horizontal bars. Gametocytemia was determined at day 7 after GlcNAc addition by analysis of Giemsa-stained smears. ‘Relative sexual conversion’ is the level of sexual conversion (%) relative to the condition in which Shld is present all the time. g, Same as in f, but GlcNAc was added simultaneously with Shld at 0–5 h.p.i. to obtain only gametocytes formed by the SCC route. For consistency with f, age is expressed as if parasites continued the replicative cycle. In f and g, values are presented as the average and s.e.m. of three independent biological replicates. h, A new model of the P. falciparum life cycle in human blood. Red circles indicate nuclear PfAP2-G expression. When PfAP2-G expression is activated early during the ring stage, gametocyte differentiation proceeds without additional replication (SCC). By contrast, when PfAP2-G is activated later, parasites go through one additional round of replication as committed forms before differentiating into gametocytes (NCC).
supported by IFA analysis over consecutive stages, which revealed a higher proportion of PfAP2-G-positive parasites in cultures at the ring or trophozoite stages than in schizonts of the previous cycle (Supplementary Fig. 12).

PfAP2-G is not essential from gametocyte stage I onwards. To identify at which stages PfAP2-G is needed for productive sexual conversion via the NCC and the SCC routes, Shld was removed from tightly synchronized E5-HA-DD cultures at different times. Under conditions promoting the NCC route, PfAP2-G stabilization was required until at least the ring stage of the second cycle (sexual ring stage) for high gametocyte production (Fig. 5f). By contrast, when conversion was restricted to the SCC route, more than half of the gametocytes could still form when Shld was removed as early as 41–46 h.p.i. of the first cycle (Fig. 5g). These results indicate that for both conversion routes PfAP2-G is no longer essential at gametocyte stage I or even earlier.

Discussion

Based on the results described here and published data, we propose an extended model for the early steps of sexual differentiation in P. falciparum. After the chromatin at the pfap2-g locus adopts a permissive state[10,18,20], either spontaneously[12,40] or induced by external cues[12,40,41], pfap2-g is transcribed mainly at the ring and late schizont stages. High expression of the gene requires a positive feedback loop involving the PfAP2-G protein. In some parasites, pfap2-g expression starts at the early ring stage and PfAP2-G levels reach a threshold sufficient to trigger the sexual transcriptional program before the onset of nuclear division, resulting in expression of early gametocyte markers and inhibition of the replicative asexual program. In such parasites, sexual conversion initiates within the same cycle of commitment as marked by PfAP2-G expression (SCC route) (Fig. 5h). By contrast, in other parasites in which PfAP2-G expression is activated, the protein levels necessary to trigger conversion are not reached early enough for SCC. We predict the existence of a checkpoint after which conversion in the same cycle is no longer possible. These parasites complete the replicative cycle as sexually committed forms, which can include at least sexually committed trophozoites and schizonts. After re-invasion, the new rings (sexual rings) have the pfap2-g locus in an active chromatin conformation inherited from the previous cycle by epigenetic mechanisms[14], and already carry nuclear PfAP2-G protein ready to drive its own expression[10,11]. Together, these conditions guarantee that high PfAP2-G levels are reached very early during the ring stage, resulting in sexual conversion one cycle after commitment (NCC route) (Fig. 5h). The observation that PfAP2-G is absent from the nucleus and dispensable from early stages of gametocyte development onwards is consistent with a role for this protein largely restricted to triggering the sexual transcriptional program, which later involves additional ApiAP2 transcriptional regulators[3,12,39].

The single evidence for the idea that an additional round of multiplication after sexual commitment is absolutely required[12,40,41] came from experiments that suggested that all merozoites from the same schizont generate only pure sexual or pure asexual clusters in plaque assays[1]. However, using an improved protocol for plaque assays with tightly synchronized cultures (see Methods for details on the modifications relative to previous protocols that can explain the different results obtained), we found that many schizonts produce only asexual or only sexual plaques, as previously observed[39], but a substantial amount of schizonts produce mixed plaques that correspond to sexual conversion events via the SCC route. The SCC route implies that PfAP2-G expression can start before nuclear replication, which was demonstrated by de novo PfAP2-G expression in rings arising from FACSt-sorted PfAP2-G-negative schizonts, and is also consistent with the temporal dynamics of basal pfap2-g expression. However, PfAP2-G expression may also be activated at different times, as shown by a recent study on induced sexual conversion demonstrating activation in late stages[37]. We cannot rule out the possibility that some changes at the chromatin or transcriptional level may already occur at the pfap2-g locus or at other loci involved in sexual commitment (for example, gvd1[2]) in individual nuclei of the PfAP2-G-negative schizonts that produce parasites that later convert via the SCC route. Future research should characterize the events that result in pfap2-g activation, identify the precise stages at which they can occur, and test the possibility that the SCC and NCC routes may reflect alternative mechanisms of pfap2-g activation, for example by determining the requirement of GVD1 (ref. 21) and the role of lysophosphatidylcholine[38] in both conversion routes. In any case, the SCC route is clearly distinct from the canonical NCC route, as it does not involve PfAP2-G-expressing schizonts or a homogeneous sexual or asexual fate for all parasites originating from the same schizont. The idea that the asexual or sexual fate of a new ring is not always irreversibly determined from the previous cycle is also demonstrated in a recently published study on sexual conversion in P. berghei[67].

New studies should establish the relative importance of sexual conversion via the SCC or NCC routes in different parasite lines, including parasites of a genetic background other than 3D7. The use of the SCC and NCC routes during natural malaria infections also remains to be determined, but it is reasonable to speculate that the existence of two alternative routes, one resulting in prompt conversion and the other amplifying the number of sexual forms, may confer an evolutionary advantage to the parasites.

Altogether, our results revise the current model for the formation of malaria transmission stages and provide a necessary framework for the design and interpretation of studies aimed at characterizing the initial molecular events involved. Of note, gametocytes are a priority target for public health interventions aiming to reduce transmission and eventually eliminate malaria[42,43].

Methods

Parasites. The 3D7 clone E5 derived from the 3D7-B stock[45], the E5-PfAP2-G–HAS3 transgenic line expressing 3xHA-tagged endogenous PfAP2-G (clone 9A, here named E5-HA), the ligand-regulatable transgenic line E5-PfAP2-G–dDFFKB expressing PfAP2-G fused to a 3xHA-tag and the FKBP-derived destabilizing domain (clone C2, here named E5-HA-DD), the 3D7 stock at Imperial College (3D7-ImP) and the gametocyte-deficient 3D7 clone F12 have been described and characterized previously[46,47]. The generation of the E5-eYFP line is described below. These parasite lines typically show sexual conversion rates of 7–15% (E5 and 3D7-ImP), 1–5% (E5-HA and E5-eYFP), 20–50% (E5-HA-DD + Shld) and 0% (F12 and E5-HA-DD without Shld). Parasites were cultured in B+ erythrocytes (3% haematocrit) under standard conditions with media containing Albamax II and no human serum. Erythrocytes were obtained from the Banc de Sang i Teixits (Barcelona) after ethical approval by the Hospital Clinic (Barcelona) ethics committee (Comitè Ètic d'Investigació Clínica). Cultures were regularly synchronized by sorbitol lysis, which eliminates erythrocytes infected with late asexual stages (trophozoites and schizonts). In some experiments, cultures were synchronized to a defined 5 h age window by purification of parasites at the schizont stage using Percoll gradients (63% Percoll) followed by sorbitol lysis 5 h later. For purification of mature parasites for transcriptional analysis in some experiments we used Percoll-sorbitol gradients (80–60–40% Percoll in the presence of 4% sorbitol) or magnetic separation using a VarioMACS magnetic separator and CS columns (Miltenyi Biotec). The PKG inhibitor ML10 (ref. 31) was used at 80 μM concentration to completely inhibit merozoite egress; it was added at the trophozoite stage (25–30 h.p.i.) and maintained until 45–50 h.p.i., when essentially all schizonts were fully mature. To stabilize PfAP2-G in the E5-HA-DD line, 0.5 μM AquaShield-I (Shld, Cheminpharma) was added to the cultures.

Sexual conversion rates were measured by treating synchronized cultures at the ring stage (5–10% parasitaemia, day 0) with 50 mM GlcNAC (Sigma) for 5–7 days to eliminate asexual parasites, approximately as described previously[48]. After treatment, asexual parasites arrest at the trophozoite stage and later die, dispensing with the need to dilute the cultures. The sexual conversion rate was calculated as the gametocytemia at days 5–7 relative to the initial ring parasitaemia at day 0. Parasitaemia and gametocytemia were measured by light microscopy analysis of Giemsa-stained smears. We measured spontaneous sexual conversion; hence, we did not intentionally stress the cultures with high parasitaemia or use any other common method for environmental induction of gametocyte formation[47,49].
Generation of the E5-eYFP line and FACS-sorting experiments. To generate the E5-eYFP line expressing endogenous PIAP2-G as a fusion protein with eYFP, E5 cultures were co-transfected with 60 µg of plasmid pDC2-Cas9-U6-hdhfr-ap2g and 12 µg of linearized plasmid pHap2g-eYFP and selected with 10 mM W909210 for 4 days as previously described. The plasmid pDC2-Cas9-U6-hdhfr-ap2g, derived from pDC2-Cas9-U6-hdhfr (ref. 40), encodes a single guide RNA that recognizes a sequence located 29–10 bp upstream from the hpaf2 g stop codon. The plasmid pHap2g-eYFP, derived from pl6-eGFP-yFUC(ref. 41), contains a homology region corresponding to the sequence immediately upstream of the Cas9 cleavage site (HR1, positions 576–788 bp from the hpaf2 g stop codon) and a homology region corresponding to a sequence downstream of the gene (HR2, positions 168–647 bp from the hpaf2 g stop codon). HR1 and HR2 flank an in-frame recombined version of the sequence between the end of HR1 and the stop codon followed by the eYFP coding sequence and the hph 903 regulatory region. The absence of cleavage of the plasmid Southern blot validation of the E5-eYFP line are provided in Supplementary Fig. 2.

E5-eYFP cultures were tightly synchronized to a 5 h age window and at 38–43 h.p.i. schizons were Percell-purified before FACS-sorting eYFP-negative parasites in a FACSArria SORP (BD Biosciences; 5 lasers, 18 parameters) cell sorter. Cultures of the parental line E5 were processed in parallel to flow a control of eYFP-negative parasites. Parasites were maintained in regular parasite culture medium at 37 °C all the time. All measurements were made using a 488 nm laser at 100 MW. The cell sorting conditions were as follows: flow chip diameter, 100 µm; sheath, solution, ISOTON (Beckman Coulter); sheath pressure, 20 psi; sort mode, purity. The erythrocyte population was identified and gated on SSC-A versus FSC-A plots. To avoid sorting of cell doublets or cell aggregates, single cells were selected on FSC-H versus FSC-A plots. E5-eYFP negative parasites were selected from plots combining fluorescence channels 488–525/30–505LP (A PIAP2-G–eYFP) and 488–582/15–556LP (for better visualization of the positive eYFP signal (Supplementary Fig. 3). Sorted eYFP-negative parasites were collected into ice-cold 1% PBS-EDTA to be processed for flow cytometry or fixed and permeabilized for IFA as described above. We used a mouse anti-Pfs16 antibody (ref. 51) (1:400–1:2,000, 32F717:B02, a gift from R. Sauerwein, Invitrogen no. A11122, lot no. 1828014; this antibody also reacts with eYFP), (1:100; Life Technologies no. 71-5500, lot no. 936618A1), rabbit anti-GFP (1:1,000; Applichem Lifescience). The conversion via the NCC route, or adding Shld just after washing out schizonts to tightly synchronized cultures, or using tightly synchronized cultures; at the time of plaque analysis asexual parasites were at the schizont stage. Flow cytometry data were collected using BD FACSDiva v.6.1.2 software and analysed using FlowJo 10.2.

Plaque assays. Plaque assays were performed following a previously described approach, but with several important modifications. The main changes, described in detail below, were as follows. (1) Overlaid schizont cultures were incubated on 1% FBS, erythrocyte monolayers were washed 10 times with 10 µM W909210 for 4 days in tight synchronization of the parasites growing in the monolayers. (2) Plaques were analysed at –40–45 h.p.i., before another round of re-invasion started. (3) To identify gametocytes by IFA we used antibodies against Pf616, which is an earlier gametocyte marker than Pf27 (ref. 42) (validated in Supplementary Fig. 4). (4) We identified the presence of the marker anti-GFP (in our assay using tightly synchronized cultures; at the time of plaque analysis asexual parasites are at the schizont stage) rather than using IFA with an anti-MS2 antibody that recognizes an antigen expressed only during a narrow age window at the very end of the asexual cycle. We analysed the number of hemozoin crystals to distinguish multineurulated parasites from multiply-infected erythrocytes. (5) In addition to the monolayers we avoided high parasitaemia in the overlaid culture to reduce the number of multiply-infected erythrocytes.

We consider that using tightly synchronized cultures and not restricting the identification of asexual parasites to a narrow age window provide especially important advantages. Using less synchronized cultures and a late schizont antigen to identify all parasites, some mixed plaques were classified as pure sexual plaques if the asexual parasites have not started expressing the marker (MS2P) yet, or entered a new replicative cycle, becoming mononucleated, MS2P-negative rings (in previous studies, plaques containing parasites not stained with either the sexual or the asexual marker were included in the analysis and classified as pure sexual). The presence of the marker anti-Pfs16 in mixed plaques may be classified as pure sexual plaques because expression of gametocyte markers is maintained for several days. A low proportion of multiply-infected erythrocytes in the overlaid culture, resulting in a low background level of mixed colonies, is also critically important.

To prepare erythrocyte monolayers, ultraviolet-sterilized coverslips were first incubated with 20 µl of 2 µg/ml ovalbumin in PBS for 30 min at 37 °C. After washing twice with RPMI HEPEs (washing medium), a 1% erythrocytes suspension in the same medium was added and incubated for 2 h at 37°C. To remove unbound erythrocytes, the dishes were carefully agitation and the medium was removed by aspiration. After two additional washes with washing medium, dishes were maintained at 37°C until parasite cultures were added.

Sorbitol-synchronized parasite cultures were grown under shaking conditions (130 r.p.m.) and at low parasitaemia to minimize the number of multiply-infected erythrocytes. The proportion of multiply-infected erythrocytes was determined at the ring stage by microscopy analysis of Giemsa-stained smears and validated by running flow cytometry performed approximately as described previously, using a FACScalibur flow cytometer (Becton Dickinson) and SYTO 11 to stain nucleic acids (Supplementary Fig. 5a). When the majority of parasites reached the schizont stage, late forms were purified using Percoll gradients. Purified schizonts were diluted to 2 × 10^4 to 8 × 10^4 mature schizonts per ml in parasite culture medium with 1.5 µl of the solution was placed on the erythrocyte monolayer. After 5 h incubation under regular culture conditions, dishes were carefully agitation and washed twice with complete parasite culture medium to remove the remaining schizonts that had not burst and re-invaded. This resulted in tight synchronization of the culture growing in the erythrocyte monolayer to a 5 h age window.

These assays were performed with the E5, 3D7-Imp. and E5-HA-3D7 parasite lines. Using 3D7 cultures were exposed under two different conditions: adding Shld approximately 18 h before Percoll purification (~30 h.p.i.) to favour sexual conversion via the RCC route, or adding Shld just after washing out schizonts from the erythrocyte monolayer (0–5 h.p.i.) to favour sexual conversion via the SCC route.

IFA analysis was performed 39–40 h after removing unbound parasites (parasite age ≥49 h.p.i.). To verify adequate progression of parasite development in the monolayers (that is, the vast majority of asexual parasites had developed to the schizont stage), we stained (with Giemsa) one of several replicate dishes. We selected for analysis preparations that had been seeded at a concentration of schizonts that resulted in widely spaced plaques (average of less than 1 plaque per field) and later when the majority of asexual parasites were again at the schizont stage. Flow cytometry data were collected using BD FACSDiva v.6.1.2 software and analysed using FlowJo 10.2.

IFAs. IFAs were performed on air-dried smears fixed for 10 min with 1% formaldehyde (Electron Microscopy Sciences) and permeabilized for 5 min with 0.1% Triton X 100 in PBS. After incubation with primary and secondary antibodies, nuclei were stained with DAPI (5 μg/ml; Applichem Lifescience). The primary antibodies used were rat anti-HA (1:100; Roche no. 11867423001; we used this antibody for all experiments except for experiments in Supplementary Fig. 12, which were performed with rabbit anti-HA antibody), rabbit anti-HA (1:100; Life Technologies no. 71-5300, lot no. 936618A1), rabbit anti-GFP (1:1,000; Invitrogen no. A10036; 130 r.p.m. and at low parasitaemia to minimize the number of multiply-infected erythrocytes. The proportion of multiply-infected erythrocytes was determined at the ring stage by microscopy analysis of Giemsa-stained smears and validated by running flow cytometry performed approximately as described previously, using a FACScalibur flow cytometer (Becton Dickinson) and SYTO 11 to stain nucleic acids (Supplementary Fig. 5a). When the majority of parasites reached the schizont stage, late forms were purified using Percoll gradients. Purified schizonts were diluted to 2 × 10^4 to 8 × 10^4 mature schizonts per ml in parasite culture medium and 1.5 µl of the solution was placed on the erythrocyte monolayer. After 5 h incubation under regular culture conditions, dishes were carefully agitation and washed twice with complete parasite culture medium to remove the remaining schizonts that had not burst and re-invaded. This resulted in tight synchronization of the culture growing in the erythrocyte monolayer to a 5 h age window.

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erythrocytes were excluded during image analysis. At the time of analysis (40–45 h.p.i.), sexual parasites were at the schizont stage. Single-nucleated parasites without hemozoin or PfS16 signal, which were observed in some of the plaques, were merozoites that did not develop in the immobilized erythrocyte monolayer and were not scored. The presence of merozoites that failed to invade and absence of ring stage parasites was confirmed by Giensa-stained smears prepared in parallel with samples for IFA analysis. A parasite was scored as sexual if it had multiple nuclei, a single hemozoin pigment and was negative for PfS16; and it was scored as asexual if it was positive for PfS16. We did not score a parasite as asexual if based on the number of hemozoin pigment signals and nuclei it may correspond to an erythrocyte infected with multiple single-nucleated parasites rather than a multicellular parasite. Of note, a very low abundance of multiply-infected erythrocytes is critically important in the overlaid schizonts culture, but multiple infection in the immobilized erythrocytes cannot be avoided and does not interfere with the identification of pure or mixed plaques.

The expected proportion of mixed plaques attributable to multiply-infected erythrocytes in the overlaid culture was calculated according to the method described previously. In brief, the calculation was based on the percentage of multiply-infected erythrocytes in the overlaid culture and the probability of a double infection producing a mixed plaque. We also calculated the expected proportion of mixed plaques attributable to multiply-infected erythrocytes among plaques containing ≥1 PfS16-positive parasites. For this calculation, the percentage of infected erythrocytes in the overlaid culture that were multiply-infected was multiplied by the probability of a schizont in the overlaid culture being asexual (according to the experimentally determined sexual conversion rate).

Transcriptional analysis. RNA for RT–qPCR was obtained using the Trizol method, DNase-treated and column-purified using a procedure optimized for low amounts of RNA. Reverse transcription was performed with AMV reverse transcriptase (Promega) using a mixture of random primers and oligo (dT). cDNAs were analysed by qPCR with the Power SYBR Green Master Mix (Applied Biosystems), using the standard curve method approximately as described previously. Transcript levels were normalized against ubiquitin-conjugating enzyme (acc; PF3D7_0812600) and siRNA synthetase (srrs; PF3D7_0771700), which show relatively stable expression across blood stages (www.plasmodb.org) and are commonly used to normalize gene expression. Primers used for RT–qPCR analysis are described in Supplementary Table 4.

Gamete egress assay. For these experiments we used the E5-HA-DD line and the parental E5 line. E5-HA-DD cultures were tested under two different conditions: adding Shld at ~30 h.p.i. and GlcNac at the next cycle (ring stage) to obtain gametocytes that mainly converted via the NCC route, or adding Shld simultaneously with GlcNac at the very early ring stage to obtain gametocytes that converted via the SCC route. In the E5 line, GlcNac was added at the ring stage. Gamete egress assays were performed essentially as described previously at days 9–15 after GlcNac addition, when the majority of gametocytes were mature (stage V). In brief, mature gametocyte cultures were incubated in complete parasite culture medium with 5 μg ml−1 WGA-Oregon Green 488 conjugate (Invitrogen no. W6748) and 2 μg ml−1 Hoechst 33342 (Sigma) for 30 min at 37 °C. Cultures were washed once with washing medium, and gametogenesis was induced by adding complete culture medium with 20 μg ml−1 xanthurenic acid (XA, Sigma) and incubating at room temperature for 30 min. As a control of non-activated gametocytes, cultures were maintained in parallel at 37 °C without adding XA. Activated gametocyte preparations were diluted in PBS with 0.75% BSA + 20 μM XA and placed in a chambered cover glass (Thermo Scientific no. 155411) for live observation on a fluorescence microscope (Olympus IX51). Images were acquired using CellSens Standard 1.11 software and processed with ImageJ software.

Western blot. To obtain total parasite protein extracts, infected erythrocytes were lysed with cold 0.1% saponin and washed with cold PBS containing protease inhibitors (Roche no. 1187380001). Isolated parasites were directly resuspended in SDS–PAGE loading buffer, boiled for 5 min at 95 °C and stored at ~80 °C. Before performing SDS–PAGE, β-mercaptoethanol was added to a final concentration of 4% and samples were boiled again for 5 min at 95 °C. Protein extracts were separated on 8% SDS–polyacrylamide gels and transferred to nitrocellulose membranes following standard procedures. We used a rat anti-HA monoclonal antibody (Roche no. 1186742001) at 1:1200 and as a secondary antibody a horseradish peroxidase (HRP)-conjugated goat-anti-rat IgG (Thermo Fisher no. A10549) at 1:1,000. To control for equal loading of parasite material between different samples, membranes were stripped with Restore stripping buffer (Thermo Scientific) according to the manufacturer’s instructions and reprobed with an antibody against PfDHFR (1:10,000; StressGen Biosciences no. SPC-186C, lot no. 1007) and an HRP-conjugated goat-anti-rabbit IgG (Sigma no. A6156) secondary antibody at 1:5,000.

Reanalysis of single-cell transcriptomic data. Single-cell analysis was carried out using the Seurat R package as described previously. Single-cell transcript counts were normalized to 10,000 transcripts per cell. Clustering resolution was chosen such that visually discretely groups of more than 100 cells were assigned to individual clusters.

Code availability. The scripts used for analysis and figure generation of single-cell RNA-seq data are available from https://github.com/KafsackLab/. Reporting Summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability. The single-cell RNA-sequencing data analysed in this study have been deposited at the NCBI Sequence Read Archive (https://www.ncbi.nlm.nih.gov/sra) under study accession code SRP16718. The authors declare that all other relevant data generated or analysed during this study are included in the Article or its Supplementary Information. Materials and protocols are available from the corresponding author on reasonable request.

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Author contributions

C.B. and A.C. conceived the project. C.B., O.L.-B. and A.C. designed and interpreted the experiments. C.B., O.L.-B., N.R.-G. and A.C. performed the experiments. A.P. and B.F.C.K. analysed single-cell RNA-seq data. A.P., C.N. and B.F.C.K. contributed resources or data. C.B. and A.C. wrote the article, with major input from O.L.-B. and B.F.C.K.

Competing interests

The authors declare no competing interests.

Additional information

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Experimental design

1. Sample size
Describe how sample size was determined.

This manuscript does not include any population-based or epidemiological research. For our lab-based experiments, we selected the number of independent biological replicates based on feasibility and variability between replicates. When differences between conditions were obvious and the patterns were consistently observed in all replicates and also in similar experiments under slightly different conditions (which could not be counted as replicates), typically we performed 2 to 4 independent biological replicates.

2. Data exclusions
Describe any data exclusions.

As described in the Methods and in the legend of Supplementary Table 1, in our plaque assays we excluded clusters that contained more than one free hemolysin pigment (residual body), because such clusters likely originated from multiply infected schizonts in the overlaid culture. This criteria was adopted during the optimization of the assays.

The 10-15h samples from one of the replicate experiments in Figure 1 d-e were lost. This is the reason why, as stated in the figure legend, data from these samples could not be included in the analysis (N=2 instead of N=3 for these time points).

3. Replication
Describe whether the experimental findings were reliably reproduced.

All findings reported could be reproduced in multiple independent experiments. The number of independent replicates is indicated in the figure legends. Findings were considered reproducible if the results of independent experiments supported the same conclusions.

4. Randomization
Describe how samples/organisms/participants were allocated into experimental groups.

N/A. This study did not involve animals or human research. All experiments were laboratory-based. None of the experiments were susceptible to randomization.

5. Blinding
Describe whether the investigators were blinded to group allocation during data collection and/or analysis.

N/A. This study did not involve animals or human research. All experiments were laboratory-based. Blinding was not suitable for any of the experiments.

Note: all studies involving animals and/or human research participants must disclose whether blinding and randomization were used.
6. Statistical parameters

For all figures and tables that use statistical methods, confirm that the following items are present in relevant figure legends (or in the Methods section if additional space is needed).

- The exact sample size \( n \) for each experimental group/condition, given as a discrete number and unit of measurement (animals, litters, cultures, etc.)
- A description of how samples were collected, noting whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
- A statement indicating how many times each experiment was replicated
- The statistical test(s) used and whether they are one- or two-sided (note: only common tests should be described solely by name; more complex techniques should be described in the Methods section)
- A description of any assumptions or corrections, such as an adjustment for multiple comparisons
- The test results (e.g. \( P \) values) given as exact values whenever possible and with confidence intervals noted
- A clear description of statistics including central tendency (e.g. median, mean) and variation (e.g. standard deviation, interquartile range)
- Clearly defined error bars

See the web collection on [statistics for biologists](#) for further resources and guidance.

7. Software

Describe the software used to analyze the data in this study.

*Graph Pad Prism v5 and v7 were used to generate the majority of graphics.
*Dropseq toolbox (including Picard tools and STAR aligner) and R were used for single cell RNA-seq analysis, including the following R packages: Seurat, Monocle, igraph, ggplot2, and rgl.
*SDS 2.4 (Applied Biosystems) was used for the analysis of real-time PCR data.
*BD Cell Quest (Becton Dickinson) was used for flow cytometry determination of parasitemia or proportion of multiply-infected erythrocytes.
*FlowJo 10.2 was used for the analysis of cell sorting data.
*CellSens Standard 1.11, LAS-AF Lite and Zen 2012 software were used for microscopy image acquisition, and ImageJ was used for image processing.

For manuscripts utilizing custom algorithms or software that are central to the paper but not yet described in the published literature, software must be made available to editors and reviewers upon request. We strongly encourage code deposition in a community repository (e.g. GitHub). [Nature Methods guidance for providing algorithms and software for publication](#) provides further information on this topic.

8. Materials availability

Indicate whether there are restrictions on availability of unique materials or if these materials are only available for distribution by a for-profit company.

All unique materials are readily available from the authors.
9. Antibodies

Describe the antibodies used and how they were validated for use in the system under study (i.e. assay and species).

All primary antibodies used in this study had been previously described (source and references provided in the Methods section) or are commercial antibodies:

- rat anti-HA (1:100; Roche #11867423001; lot number not available);
- rabbit anti-HA (1:100; Life technologies #71-5500, lot #936618A1);
- rabbit anti-GFP (1:1,000; Invitrogen #A11122, lot #1828014; this antibody also reacts with eYFP);
- mouse anti-Pfs16 (1:400-1:2,000, 32F717-B02, a gift from Robert Sauerwein, Radboud University). Reference 51 of the article;
- mouse anti-Pfg27 (1:2,000, 482, a gift from Richard Carter, University of Edinburgh). Reference 23 of the article;
- rabbit anti-H3K4me3 (1:10,000; Merck-Millipore #04-745, lot #DAM1606783);
- rabbit anti-H3K9me3 (1:1,000; Merck-Millipore #07-442, lot #DAM1810831).

The secondary antibodies used were:

- goat-anti-rat IgG conjugated with Alexa Fluor 488 (1:1,000, Thermo Fisher #A11006, different lots);
- goat-anti-mouse IgG - Alexa Fluor 594 (1:1,000, Thermo Fisher #A11007, different lots);
- goat-anti-mouse IgG - Alexa Fluor 488 (1:1,000, Thermo Fisher #A11029, different lots);
- donkey-anti-mouse IgG - Alexa Fluor 546 (1:1,000, Thermo Fisher #A10036, different lots);
- goat-anti-rabbit IgG - Alexa Fluor 594 (1:1,000, Thermo Fisher #A11012, different lots).

As stated in the Methods section, our validation of the specificity of the antibodies included absence of signal at parasite stages in which the protein is not expressed, and in the case of antibodies against an artificial 3xHA or eYFP tag comparison with the level of signal in wild type parasites that do not carry the tag (analyzed at the same stage of the life cycle). For anti-Pfs16 and anti-Pfg27 antibodies, additional characterization of the temporal dynamics and specificity of the signal is presented in Supplementary Fig. 4.

For commercial antibodies against the histone post-translational modifications H3K9me3 and H3K4me3, validation includes coincidence with the previously reported distribution of heterochromatin and euchromatin within P. falciparum nuclei, and in the case of the antibody against H3K9me3 also previous reports of ChIP analysis with these antibodies that gave the expected distribution of the signal (e.g. Crowley et al. 2011, PMID: 21306446; López-Rubio et al. PMID: 19218088). Additionally, the company (Merck-Millipore) provides the following statements:

- Rabbit anti-H3K9me3 #07-442: Peptide Inhibition: Specificity was confirmed by the ability of 10 mM of the immunizing peptide to completely abolish detection of histone H3 in immunoblot analysis of HeLa acid extracts. No inhibition of detection was observed by preabsorption of the antibody with 10 mM histone H3 peptide containing mono- or dimethyl-lysine 9, or mono-, di- or trimethyl-lysine 27 modifications (Data not shown). Western Blot Analysis: 1:500 dilution of this lot detected trimethyl Histone H3 on 10 μg of HeLa acid extract but not on recombinant Histone H3.
- Rabbit anti-H3K4me3: routinely evaluated by immunoblot on acid extracted proteins from HeLa cells, but not recombinant unmethylated Histone H3.
10. Eukaryotic cell lines

a. State the source of each eukaryotic cell line used. All Plasmodium falciparum lines used in this study are described in the first section of the Methods. Their sources are:

- 3D7 subclone E5 (derived from the 3D7-B stock), ES-PAP2-G–HAx3 transgenic line expressing 3xHA-tagged endogenous PfAP2-G (clone 9A, here named E5-HA), and E5-eYFP transgenic line expressing eYFP-tagged endogenous PfAP2-G: generated in our laboratory;
- ligand-regulatable transgenic line ES-PAP2-G–ddFKBP expressing PfAP2-G fused to a 3xHA-tag and the FKBP-derived destabilizing domain (clone C2, here named E5-HA-DD): obtained from Manuel Llinás (Pennsylvania State University, USA);
- 3D7 stock at Imperial College (3D7-Imp.): obtained from Michael J. Delves (Imperial College, UK);
- gametocyte-deficient 3D7-subclone F12: obtained from Pietro Alano (Istituto Superiore di Sanità, Italy).

b. Describe the method of cell line authentication used. The parasite lines used were not directly authenticated, but their sexual conversion rates, pfap2-g transcript levels and expression of an exogenous 3xHA/eYFP tag or a destabilization domain were always consistent between experiments and with previous published reports. This somehow serves as a "phenotypic" authentication. Additionally, the E5-eYFP line was regularly monitored by PCR analysis to validate presence of the sequence encoding the tag and absence of wild type parasites.

c. Report whether the cell lines were tested for mycoplasma contamination. The parasite line E5-HA-DD (C2 clone) tested positive for mycoplasma contamination in a PCR-based test, whereas other parasite lines tested negative. However, mycoplasma contamination is not believed to affect any of the parameters analyzed in this study.

d. If any of the cell lines used are listed in the database of commonly misidentified cell lines maintained by ICLAC, provide a scientific rationale for their use. N/A. None of the cell lines used in this study (all P. falciparum) are listed in the database of commonly misidentified cell lines.

Animals and human research participants

Policy information about studies involving animals; when reporting animal research, follow the ARRIVE guidelines

11. Description of research animals

Provide details on animals and/or animal-derived materials used in the study.

No animals were used.

Policy information about studies involving human research participants

12. Description of human research participants

Describe the covariate-relevant population characteristics of the human research participants.

This study did not involve human research participants.