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An ApiAP2 transcription factor influencing virulence gene transcription and sexual development in *Plasmodium falciparum*

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Abstract

The human malaria parasite *Plasmodium falciparum* expresses variant PfEMP1 proteins on the infected erythrocyte, which function as ligands for endothelial receptors in capillary vessels, leading to erythrocyte sequestration and severe malaria. The factors that orchestrate the mono-allelic expression of the 50-60 PfEMP1-encoding *var* genes within each parasite genome are still not fully identified. Here, we show that the transcription factor PfAP2-O influences the transcription of *var* genes and other multigenic families. The temporary knockdown of PfAP2-O leads to a complete loss of *var* transcriptional memory and a decrease in cytoadherence. AP2-O-knocked down parasites exhibited also significant reductions in transmission through *Anopheles* mosquitoes. We propose that PfAP2-O is one of the major virulence gene transcriptional regulators and may therefore be exploited as an important target to disrupt severe malaria and block parasite transmission.

Introduction

The human malaria parasite *Plasmodium falciparum* remains a risk to a significant portion of the world's population. Despite the considerable success of control measures, *P. falciparum* still causes around 400000 deaths per year, mainly in the sub-Saharan African countries, where most of the victims are either pregnant women or children under 5 years of age [1]. Common approaches for malaria control and elimination are artemisinin-based combination therapies (ACT) to treat blood stages in infected

patients, and insecticide-impregnated bed nets to prevent mosquito bites and parasite transmission. Although a significant reduction of malaria cases during the first decades of implementation of the aforementioned measures was achieved, recent studies have demonstrated the emergence of parasites resistant to almost all antimalarial treatments including artemisinin [2], and of mosquitoes' resistance to the most commonly-used insecticides – pyrethroids, organochlorines, carbamates, and organophosphates – across different endemic areas [3]. Therefore, the study and characterization of new drug targets to interfere in parasite transmission are crucial to achieve long-term malaria eradication [4].

A major parasite virulence factor is PfEMP1 (*Plasmodium falciparum* erythrocyte membrane protein 1), which consists of proteins expressed on the infected red blood cell surface, encoded by the multicopy *var* gene family [5]. The *var* genes are expressed in a timely, controlled manner, so that only one or a few *var* genes are transcribed per parasite, which prevents immune clearance by immune responses directed against any single PfEMP1 variant [67]. Different factors are involved in *var* gene transcription, culminating in a process known as allelic exclusion. Specific sequence elements found in the promoter region recruit *var* 5' upstream regions resulting in co-regulation with other *var* loci [8]. The *var* intron found in all complete *var* genes appears to bi-directionally transcribe non-coding RNAs which are involved in *var* promoter activity [910], although activation or silencing can also be achieved in the absence of this motif [11]. GC-rich ncRNAs seem to play a major role in the control of *var* gene expression [12,13], and the exosome related RNAse PfRrP6 seems to fine-regulate the degradation of these RNAs [14] . Several proteins interact directly or indirectly with *var* 5' upstream regions: Heterochromatin protein 1 (HP-1) is associated with silenced chromatin around the promoter region of *var*, and also with with genes involved in the production of sexual/transmission stages of the parasite, known as gametocytes [15]. Accordingly, HP-1 knockdown leads to transcriptional de-repression of *var* genes, disruption of allelic exclusion, and the induction of gametocytogenesis, indicating a link between *var* gene transcription and transmission. Several histone modifications, mostly of lysins in histone 3, can be associated with either activated or silenced *var* loci [16]. A specific DNA helicase, RecQ1, associates with actively-transcribed *var* loci [17]. Based on features such as chromosomal location, protein domain structure and sequence similarities, *var* genes have been classified into three major groups, termed upsA, upsB and upsC [18] that seem to be subject to specific mechanisms of regulation; for example, the activity of a particular RNAse II controls transcription of the upsA subgroup [19].

Members of the ApiAP2 family of plant-like transcription factor proteins have been associated with important processes throughout the entire life-cycle of *Plasmodium*. In the rodent malaria parasite *P. berghei,* parasites lacking the protein encoded by *PbAP2-Sp* (PF14_0633/PF3D7_1466400

P. falciparum ortholog) were unable to complete the sexual cycle inside the mosquito, as they could not form sporozoites [20]. *P. berghei* parasites lacking *PbAP2-O* (Pf11_0442/PF3D7_1143100 *P. falciparum* ortholog) developed as aberrant ookinetes that were unable to invade the *Anopheles* midgut and form oocysts [21]. In *P. falciparum,* the disruption of *PfAP2-G* (PF3D7_1222600) resulted in a complete loss of gametocyte formation, whereas increased expression led to the increased induction of gametocyte-commitment [22]. Campbell and colleagues identified domains in three ApiAP2 members [23] that showed affinity to specific domains in *var* 5' upstream (5' ups) regions [24]. One of them (PF3D7_0604100/PfSIP2) was shown to interact with upsB *var* sequences, and it was suggested that this protein has a role in tethering of chromatin [25]. For the second gene, Martins and colleagues demonstrated an upregulation of clonally-variant genes (*stevor* and *rif*, but not *var*) when the predicted protein, PF3D7_1466400/ApiAP2-exp was truncated [26]. The role of the third protein, PF3D7_1143100/Pf11_0442, remains unknown in *P. falciparum*. Here, we addressed the function of this protein, named PfAP2-O, in the asexual and sexual cycle of *P. falciparum* and found that it influences transcription of multiple genes including variant gene families and is crucial for parasite transmission to *Anopheles* mosquitoes.

Results

Decreased quantities of PfAP2-O do not interfere with *P. falciparum* blood-stage growth

NF54 parasites were successfully modified by single crossover recombination to contain a destabilizing DD24 domain [27] in 3'-position of the *PfAP2-O* open reading frame (Figure 1). Removal of Shield-1 from the cultures led to an ~85% depletion of the corresponding protein in mutant parasites (Figure 1, and Supplementary Table 1). As expected from knockout experiments of the orthologous gene in *P. berghei*, the silencing of this factor did not result in a growth defect after 96 h in the absence of Shield-1 compared to control parasites (Figure 1). This shows that PfAP2-O can be tagged with additional domains and its depletion has no immediate influence on asexual stage parasite survival. Using the GFP-tag in this transgenic strain, we observed a nuclear localization of the protein, exclusively in schizonts (Figure 2).

PfAP2-O knockdown leads to diversified transcription number of *var* genes

Recombinant AP2-domains of PfAP2-O were predicted to bind to elements found in the 5' ups regions of *var* genes [24]. To elucidate any influence of PfAP2-O on *var* transcription, we selected clonal parasite populations to dominantly express a single dominant *var* gene (*PF3D7_0412400*) by repeated panning of PfAP2-O-GFP-DD24 trophozoite/schizont-infected RBCs over CHO-CD36 cells, as described previously [28] (Figure 3A). After this, we performed the silencing of the PfAP2-O for 96 hours, followed by re-establishment of the protein to observe *var* gene transcripts during knockdown and after reestablishment of PfAP2-O. RNA samples from ring stage-parasites were collected from three

differently treated cultures: 1. RNA from panned parasites (initial sample), 2. RNA from parasites with PfAP2-O knockdown by the absence of Shield-1 for two reinvasions, and 3. RNA from parasites two reinvasions after the knockdown and reestablishment of PfAP2-O by addition of Shield-1 (Figure 3).

In the absence of Shield-1, several *var* transcripts showed increased relative amounts (*PF3D7_0100300, PF3D7_0412900, PF3D7_0420700, PF3D7_0500100, PF3D7_0533100, PF3D7_0711700, PF3D7_0800100, PF3D7_0900100, PF3D7_0937600, PF3D7_1041300*) while the initially dominant *var* gene transcript *PF3D7_0412400* was less abundant (Figure 3B). When checking for chromosomal location of the differently transcribed *var* genes, we observed transcripts from all *var* subgroups, upsA, upsB and upsC. Furthermore, while most of the differentially transcribed *var* genes showed the 5' ups recognition motifs identified for subdomains of PfAP2-O [24], others did not, such as *PF3D7_0937600* and *PF3D7_0800100*. Thus, the depletion of PfAP2-O exerts mostly a de-repressing effect on *var* loci that were not exclusively related to any *var* gene subgroup. Importantly, the continuous Shield-1 treatment itself does not alter *var* transcription or *var* transcription memory, since wildtype NF54 and Shield-1-treated transfectant parasites showed similar *var* transcription patterns after selection on CHO-CD36 cells (Figure 3 C, and data for wild type parasites not shown).

Short-term silencing of PfAP2-O leads to the complete deletion of *var* transcription memory

The *var* transcription memory over multiple reinvasions is believed to be maintained by factors that direct chromatin readers and writers to their respective sites of action. Transcriptional activity of genes is reversibly determined in the specific histone code of methylation and acetylation of histone 3 lysins and perhaps others [29]. If PfAP2-O is acting hierarchically upstream in the events that result in the recruiting of silencing and activating factors, then its knockdown should erase the epigenetic memory of *var* gene transcription. To verify what influence a temporary knockdown of AP2-O had on *var* transcription, we observed the *var* transcript profile when the protein function was re-established by re-adding Shield-1. The pre-silencing predominant active locus *PF3D7_0412400* appeared to be silenced after reestablishment of PfAP2-O. Instead, transcripts from its neighbouring locus *PF3D7_0412700* and from upsA *PF3D7_0400400* were dominantly detected, indicating that the transcriptional *var* memory had been erased. In comparison, cytoadherence-selected parasites that were cultivated on Shield-1 throughout the experiment were still dominantly expressing *var* gene *PF3D7_0412400*, confirming that no spurious global switching event occurred during the growth period (Figure 3C and Supplementary Figure 1).

Depletion of PfAP2-O activates gametocyte-related genes

Several members of the ApiAP2 family have been associated with important processes in the *Plasmodium* life cycle, such as gametocyte commitment (*PfAP2-G/PfAP2-G2*)[22,30], liver stage [31], sporozoite (*PbAP2-SP*)[20] and oocyst (*PbAP2-O*)[21] development, and invasion ligand transcription

(*PfAP2-I*) [32]. Considering its role in the transformation to ookinetes in *P. berghei*, we tested if the knockdown of PfAP2-O regulated any of the gametocyte-related transcripts induced by PfAP2-G as well as the transcript of PfAP2-G itself. Interestingly, the PfAP2-O silencing had a mixed effect on gametocyte-associated transcripts in trophozoite stage parasites. While no difference was discernible in the steady-state transcript quantities of PfAP2-G (*PF3D7_1222600*), several key gene transcripts previously associated with gametocyte development appeared in increased amounts (Figure 4A). The transcript of the early gametocyte-expressed gene *Pfs16* (*Pf3D7_0406200*) appeared increased up to 4-fold when compared with the control. When the PfAP2-O protein was re-established, we observed that most of the transcripts returned to previous expression levels, with the exception of Pfs16, which remained at high levels. To investigate if the Pfs16 transcript was indeed being translated, its corresponding protein was detected with a specific antibody. While during PfAP2-O-GFP knockdown Pfs16 was readily detected, re-establishment of PfAP2-O-GFP (Figure 4B) resulted in the disappearance of the protein. Thus, PfAP2-O may also be involved in the regulation of translation of at least Pfs16.

Massive parallel transcript analysis identifies targets of PfAP2-O

Other transcripts may be influenced during temporary silencing of PfAP2-O. To address this, we performed an RNA-seq analysis and compared the genome-wide transcriptional profile under three different conditions, exactly as was done with the *var* analysis. We detected 512 upregulated transcripts when the PfAP2-O protein was depleted by Shield-1 removal. Among them, several members of the multigenic families *rifin*, *stevor* and *surfin* as well as alleles of the PHIST family were identified (Figure 5). We also observed 101 downregulated genes, including the early gametocyte marker *PF3D7_0936600.1/PfGEXP5*, another AP2 domain containing protein (*PF3D7_0934400*), a bromodomain containing protein (*PF3D7_1212900*) and the translocon component *PTEX88* (*PF3D7_1105600*) (Supplementary Table 2).

Furthermore, we observed a reversal of this effect in PfAP2-O-reestablished parasites since most of the initially upregulated *rifin*, *stevor*, *surfin,* and *phist* transcripts returned to pre-silencing levels. Of the 512 transcripts that were significantly more abundant during knockdown of PfAP2-O, only 16 remained significantly more abundant after PfAP2-O reestablishment, while only four transcripts remained less abundant than the control sample. The *surf* transcripts *PF3D7_1301800.1/Surfin 13.1* and *PF3D7_0402200.1/Surfin 4.1* were still present in higher quantities when AP2-O was re-established. Also, a *rifin* member (*PF3D7_0401600*) that was not altered during Shield-1 removal, was upregulated in PfAP2-O-reestablished parasites (Supplementary figure 2, and Supplementary table 2), indicating transcriptional switching. Therefore, the transcriptional profile of other multigenic families also changed during and after temporary PfAP2-O silencing, as observed by qPCR for *var* genes. Using tools available on plasmodb.org we searched for the predicted PfAP2-O binding motifs 1000 nucleotides upstream of the coding regions of all differentially regulated genes in

the control samples versus the knocked down samples and identified 49 with these target motifs, including members of *var, surfin* and *rif* (Supplementary Table 3) and PfAP2-O itself. Of note, the PfAP2-O transcript is slightly but significantly more abundant in parasites under PfAP2-O protein knockdown (Supplementary Table 2).

In global terms, most of the transcripts that were more abundant in PfAP2-O knocked down parasites compared to control samples, were also significantly less abundant upon recovery and reestablishment of PfAP2-O (n=372). Similarly, of the 101 transcripts that were less abundant upon knockdown of PfAP2-O, 55 were significantly more abundant in PfAP2-O re-established parasites (Supplementary Table 2), and only four remained less abundant after recovery. This shows a reversible modification of multiple transcripts dependent upon the presence or partial absence of PfAP2-O, but also a delayed effect in the case of transcripts that did not return to initial quantities.

Absence of PfAP2-O modifies the cytoadherence phenotype

A landmark of different types of severe malaria is the adherence of infected red blood cells (IRBC) to determined receptors. Considering the observation that the PF3D7_0412400 *var* transcript was much less abundant in a transient knockdown of PfAP2-O, we tested if the parasites also lost the CHO-CD36 adherent phenotype. For this, a static cytoadherence procedure was conducted during and after knockdown. Both CHO-CD36 cells and parasites were counted (Figure 6). We observed that during and after silencing of PfAP2-O the number of parasites adhering to CHO-CD36 cells was significantly lower than the initially CHO-CD36 selected parasites and also the control parasite line, indicating that this factor may influence *P. falciparum* antigenic variation mediated by PfEMP1 proteins.

PfAP2-O interacts with histone modifiers

PfAP2-O probably interacts with other factors to exert its biological role. To identify proteins that physically interact with PfAP2-O, a total protein extract was prepared and the protein complex was immunoprecipitated via the HA tag and purified. Three protein bands at molecular weights of 70, 30 and 15 kDa (which did not appear in NF54 wildtype extracts immunoprecipitated in parallel) were detected and analyzed by mass spectrometry (Supplementary Figure 3). In all of these extracts, fragments of a 167 kDa protein appeared and this protein (PF3D7_0216700/PFB0765w) was predicted to be related to autophagy. Fragments of an essential 402 kDa protein with unknown function (PF3D7_0317300/PFC0650w) was detected in the 70 kDa and the 15 kDa fractions. We also observed that PfAP2-O associated with several histone modifiers such as histone-lysine N-methyltransferases SET1 (encoded by *PF3D7_0629700*) and SET2 (encoded by *PF3D7_1322100*, PfSET2/PfSET-VS) and another member of the ApiAP2 family (*PF3D7_0420300*, Supplementary table 4). Intriguingly, no peptides from histones were identified in this assay, indicating that PfAP2-O may not directly interact with these.

PfAP2-O is essential for *P. falciparum* sexual development

The *P. berghei* AP2-O protein (PbApiAP2-O) was associated with mosquito transmission, since knockout parasites for this protein could not form functional ookinetes capable of invading the *Anopheles* midgut and thus completing the sporogonic cycle [21]. We then asked whether the PfAP2-O protein would have the same effect in *P. falciparum*. For this, *Anopheles coluzzii* mosquitoes were fed with the PfAP2-O-GFP-DD24 strain without Shield-1 (AP2-O *knockdown*, KD) and the prevalence of infected mosquitoes and oocyst number was measured and compared with the NF54 WT strain. In all experimental feedings, the infection prevalence was lower than 10% for the KD strain and higher than 40% for the WT (Figure 7 A). Using a generalized linear mixed model (GLMM) (Supplementary file 1) to predict the effects of PfAP2-O knockdown on parasite transmission, we observed a significantly lower oocyst prevalence in mosquitoes infected with thePfAP2-O knockdown line compared to the NF54 wild type (P-value: $2.6*10^{-6}$), controlling for gametocyte numbers in the infectious blood meal which were positively correlated with infection (P=0.0158). The odds ratio values indicated that mosquitoes fed blood meals containing knockdown parasites had a significantly lower probability of infection than those fed with the WT parasites (OR: 0.09). We also observed that a higher number of gametocytes in the infectious blood meal correlated with increased chances of infection (OR: 1.56).

When the intensity of the infection (oocyst numbers) in mosquitoes was analysed, we observed that in all experiments, the number of oocysts in mosquitoes fed with KD parasites was significantly lower compared to those fed with the NF54 WT parasites (Figure 7 C). In a GLMM analysis, infection intensity (Supplementary file 1) was significantly influenced by the parasite clone used ($P=5.61*10^{-14}$), but not by the gametocyte number in the blood meal (P: 0.0541) (Figure 7 D).

These data show that the knockdown of PfApiAP2-O significantly decreased both the prevalence and the intensity of infection in mosquitoes, and predicts a positive relation between the expression of the protein and the transmission of *P. falciparum* from human to *Anopheles* mosquitoes.

Discussion

Transcriptional regulation in *Plasmodium* is typically related to chromatin modification and in this regard, histone methyltransferases, demethylases, histone acetyltransferase and deacetylases have a central role [33,34]. In the case of *var* genes, however, the hierarchy of events that finally leads to the selective activation of one promoter and the inactivation of the previously active one (switching) is unclear, as is the participation of other factors besides chromatin modifiers. During asexual growth, the histone modification H3K4me2 is known to mark promoters temporarily inactive in trophozoites and schizonts for transcription after the next reinvasion [29]. Probably just before transcription, a DNA helicase – RecQ1 – is recruited to the to-be-activated *var* site [17], perhaps mediated by an interaction of factors loaded with antisense ncRNAs stemming from the promoter activity localized in the *var*

intron [9,10,35] or with GC-rich ncRNAs [13]. HP1 is found at transcriptionally silenced sites in the genome and is believed to recruit deacetylases and methyl-histone transferases [15], which then modify *var*-associated chromatin (H3K9me3), by the action of at least SIR2A/B [33,36]. For a number of upsA *var* loci, a special RNAse, PfRNAse II, plays a role in silencing upsA derived transcripts [19]. The concentration of ncRNA RUF6 from different loci seems to play a general role in silencing heterochromatic genes such as *var, rif* and others, given that the knockdown of the RNAse PfRrP6 leads to generalized de-repression of heterochromatin [14]. We hypothesized that by using temporary knockdown of factors putatively participating in these events, the hierarchy of factors playing a role in maintenance or modification of epigenetic signatures of *var* transcription might be elucidated.

Here, we show that PfAP2-O, in contrast to its orthologue PbAP2-O, is expressed in schizont stage parasites, already indicating additional roles besides the control of expression of female gametocyte- and ookinete-related genes [21]. Yuda and colleagues showed a translational repression of the PbAP2-O transcript by PbDOZI, which is not the case for the *P. falciparum* orthologue, since it is clearly expressed in schizonts. Intriguingly, but possibly due to its low abundance, PfAP2-O was also not detected in the nuclear proteome of either ring, trophozoite or schizont stages [37]. Similarly to PbAP2-O, PfAP2-O was also not essential for asexual proliferation, and long-term growth in the absence of Shield-1 revealed no change in parasite development or growth rate during the asexual intraerythrocytic cycle.

The knockdown of PfAP2-O led to a swift and complete change of *var* transcription patterns, indicating that PfAP2-O is decisively involved in maintaining the transcriptional memory and acts as an important factor in *var* switching. Based on the results of previous work [29,38,39], we hypothesize that histone lysine 9 modifications (trimethylation or acetylation) are different at *var* loci before and after knockdown of PfAP2-O. In consequence, PfAP2-O seems to guide mostly inactivating, chromatin-modifying factors to loci, since in the absence of PfAP2-O, a number of *var* and *rif* loci - but not all - are actively transcribed. It is intriguing that the transcript analysis revealed that the same *var* loci were activated in three biological replicates. Thus, it appears that parasites with activated *var* locus *Pf3D7_0412400* preferentially switch to the same *var* loci identified above when PfAP2-O is temporarily unavailable or available in decreased quantities. Interestingly, when the predicted binding motifs for PfAP2-O were explored in the deregulated genes, only 49 possessed the binding motif (Supplementary table 3.1). A similar observation was described for PfAP2-exp, another member of the ApiAP2 family that was also associated with the control of several gene families [26]. These data suggest that the binding predictions of motifs for partial AP2 domains may be different from the binding activities of the respective holoproteins.

The change of *var* transcription was also reflected in the change of cytoadherence patterns in parasites subjected to PfAP2-O protein depletion. Here, IRBC under PfAP2-O knockdown adhered significantly less to CHO-CD36 cells, probably due to the virtual disappearance of the PfEMP1 that most effectively binds CD36, encoded by *Pf3D7_0412400*. Taking into account that IRBC binding to CHO-CD36 occurred in fact via CD36 and not via other unrelated receptors found on CHO cells [40], then interaction with the surface of CHO-CD36 cells of the PfEMP1 ectodomain present in Pf3D7_0412400 is apparently stronger than that of the PF3D7_0412700 ectodomain. Both PfEMP1 ectodomains were predicted to be competent for CD36 binding, while the second most transcribed *var PF3D7_0400400* encodes a CIDRα1.1 domain, which is binding to CD36 [41]. The *var PF3D7_0400400*, upregulated after knockdown and recovery of PfAP2-O, is the so-called var_{severe} [42] of the upsA *var* group of which expression is upregulated in severe malaria cases [43] and in the onset of infection in non-immune individuals [44]. In endemic areas, the disruption of cytoadherence remains a challenge in the treatment of patients with severe malaria [45]. The fact that the plant like ApiAP2 proteins are not encoded in the human host may point PfAP2-O to a novel target of intervention to block the adhesion of infected red blood cells, possibly alleviating life-threatening cytoadherence patterns.

Histone protein 1 (HP-1) has previously been shown to control transcription of gametocyte-associated genes by de-repression of *PfAP2-G*, but also that of variant genes such as *var*, *rif* and *Pfmc-2TM* [15]. Here, we demonstrated a similar effect with the PfAP2-O factor, which appears to be independent of HP1, since no association between PfAP2-O and HP1 was observed by transcriptomic or proteomic analysis. Based on the upregulation of *var, rif, stevor, PHIST* and *surfin* genes, as well as some gametocyte-associated genes when the PfAP2-O is depleted, we hypothesize that PfAP2-O exerts a repressing effect on several multigenic families and gametocyte markers. These observations underscore the role of this factor in transcriptional memory, not exclusively for *var* genes, but also its involvement in the control of other gene families. PfAP2-O could promote the recruitment of HP-1 to specific sites, which then leads to gene silencing; in the absence of PfAP2-O, the HP-1-guiding effect would then be impaired at least for a number of loci. The role of PfAP2-O in variant gene control is further supported by the observation that different histone modifiers such as PfSET1 and also PfSET2 co-precipitated with PfAP2-O, since these have already been linked to *var* transcription [46]. Importantly, the transcripts of other DNA and RNA binding factors (Supplementary table 2) were differentially present in parasites with knocked-down PfAP2-O and these may play roles in the effects observed here.

Regarding sexual commitment, we detected the increased presence of several early gametocyte-related transcripts in PfAP2-O knocked-down parasites. Transcription of the early gametocyte gene *Pfs16* (*Pf3D7_0406200*) was strongly increased upon PfAP2-O knockdown, but did

not return to pre-knockdown levels after re-exposure of the parasites to Shield1. This may indicate the passing of some as-yet undiscovered checkpoint that leads to persisting *Pfs16* transcription. However, despite the persistence of *Pfs16* transcript, Pfs16 protein was no longer detected after re-establishment of PfAP2-O, suggesting additional post-transcriptional control to present translation of this protein when PfAP2-O is present. Of note, post-transcriptional control of Pfs16 has been previously described [47], but translational repression of Pfs16 seems not to be influenced by the RNA helicase DOZI [48]. This suggests that other RNA helicases or factors are involved in the regulation of Pfs16 translation, and that these are directly or indirectly influenced by PfAP2-O.

Commitment to gametocyte development is known to be controlled by another member of the ApiAP2 family, PfAP2-G/PF3D7_1222600 [22]: Increases in PfAP2-G leads to increased transcription of gametocyte-related genes and conversion to gametocytes. The increased expression of PfAP2-G had no significant influence on the transcription of *PfAP2-O* [49], conversely, we observed that knockdown of PfAP2-O had no enhancing effect on the presence of the *PfAP2-G* transcript. Of note, the earliest gametocyte marker detected so far, gametocyte development protein 1 (GDV-1, [50]), was slightly but significantly downregulated in PfAP2-O knockdowns, reinforcing that PfAP2-O plays no decisive role in gametocyte induction.

We also investigated the role of PfAP2-O in parasite transmission through mosquito infection. There was no difference in the number of mature gametocytes obtained under standard culturing conditions for the WT and KD clones (supplementary Figure 4). Furthermore, the gametocytes produced by the PfApiAP2-O knocked down line did not present any difference in shape or size compared to the NF54, which indicates that this protein does not play an important role in gametocyte commitment, however is still uncertain whether these gametocytes have a biological defect that blocks the fertilization and mosquito midgut invasion. In these experiments, we did not study fertilization and ookinete formation to assess this question due to the difficulties to induce them *in vitro* in *P. falciparum*. Our results showed that mosquitoes fed with the PfApiAP2-O strain under knockdown had a lower level of infection than those fed with the NF54 WT control, similar to the results shown for the PfAP2-O ortholog in *P. berghei* [21]. We showed that protein depletion of PfApiAP2-O led to a significant decrease in the prevalence of infected mosquitoes as well as a reduction of oocyst numbers. Thus, PfAP2-O appears to be essential for mosquito invasive stages, as was shown for its ortholog in *P. berghei* [21]. These observations suggest a conservative role of this protein in human-mosquito transmission among *Plasmodium* species. Finally, the ApiAP2-O knockdown did not result in complete blockade of mosquito infection: a minority of mosquitoes still was infected, with very low oocyst numbers. This could be explained by the fact that the knockdown is not complete and small amounts of protein are detected during Shield withdrawal.

Interactions between ApiAP2 proteins in *P. berghei* are involved in crucial processes during the parasite life cycle, including gametocyte development, and oocyst and sporozoite formation [51]. In our proteomic co-immunoprecipitation analysis another ApiAP2 member, *PF3D7_0420300/PFD0985W*, with no defined function, was also detected. Campbell and colleagues identified a significant number of target genes predicted for this protein, mainly associated with DNA replication and gene expression [24]. When binding site evidence for this factor were analyzed in differentially regulated genes in the PfAP2-O knockdown background, we observed 106 targets (more than twice the number that was observed for PfAP2-O) with at least one binding motif (Supplementary Table 3), most of them associated to the cellular processes mentioned by Campbell and colleagues. This evidence leads us to hypothesize that these members may be part of a protein interaction network responsible for important steps in *P. falciparum* life cycle, similar to that described in the murine parasite model.

In conclusion, we showed that PfAP2-O controls transcript levels of clonally variant gene families, antigenic variation and transcriptional memory in *P. falciparum* asexual cycle. We also demonstrated a significant decrease in *P. falciparum* transmission to *Anopheles* mosquitos when this protein is absent. PfAP2-O probably interacts with other transcription factors and histone modifiers in a dynamic protein complex involved in several key processes in *P. falciparum* biology. These results highlight PfAP2-O as an attractive drug target to alleviate virulence factor-induced pathogenesis, with additional impacts through blocking the transmission of the parasite from infected human to the invertebrate host.

Figures

Figure 1

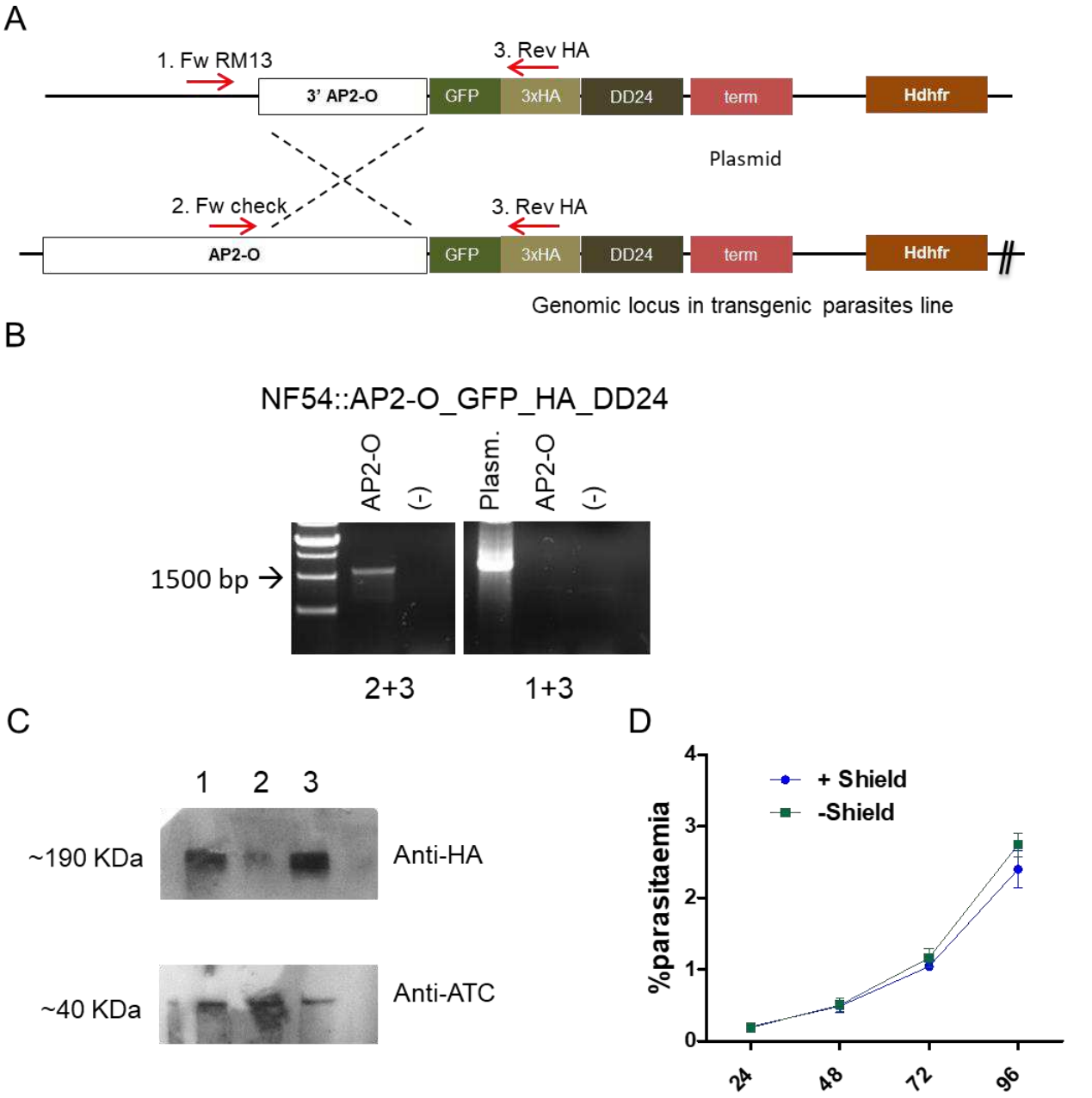

Figure 1. PfAP2-O modification and subsequent knockdown leads to no discernible growth phenotype. **A**: The plasmid structure and its integration of tags by homologous recombination after transfection and cloning is shown. **B**: PCR analysis to confirm the correct integration of the plasmid. The oligonucleotides indicated in **A** resulted in the presence of the expected amplicons. Amplicons using oligo pair 2+3 indicate successful integration, while products using pair 1+3 indicate the presence of episomal forms. C: Western blot using anti-HA antibody to detect PfAP2-O-GFP expression in parasites submitted to temporary knockdown by the absence of Shield-1. Lane 1: Parasites grown in the presence of Shield-1, Lane 2: Parasites grown for two reinvasions without Shield-1, lane 3: Parasites after re-establishment of PfAP2-O-GFP by re-addition of Shield-1 for two reinvasion cycles. As a loading control, a polyclonal antiATC (plasmodial aspartate carbamoyltransferase) was used. **D:** Growth curves from NF54::PfAP2-O_GFP_HA_DD24 with and without 0.5 µM Shield-1 (data from triplicates)

Figure 2

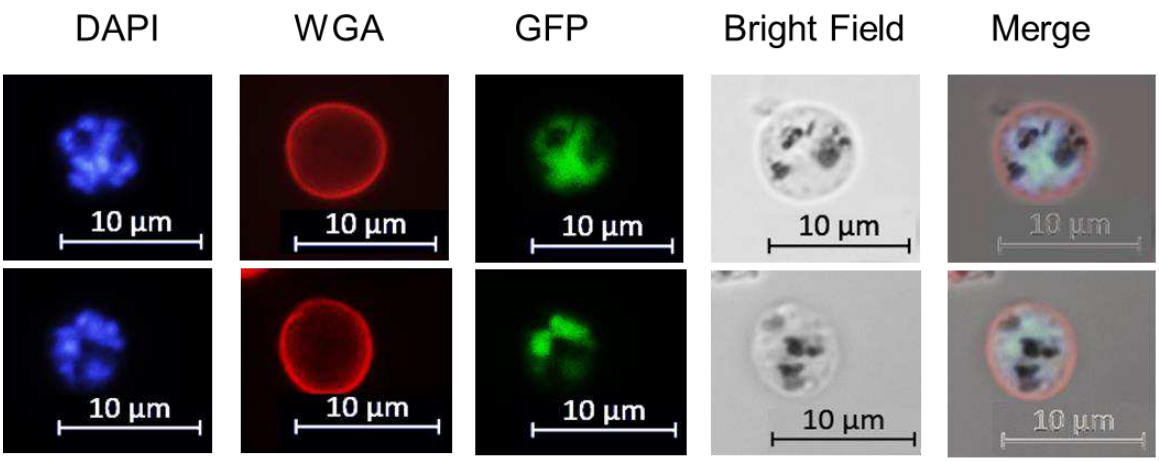

Figure 2: PfAP2-O-GFP-HA-DD24 is expressed in schizont stage parasites. A: Fluorescence microscopy of late schizont parasites shows that GFP-tagged PfAP2-O colocalizes with the DAPI (nuclear stain) signal. WGA marks the surface of red blood cells.

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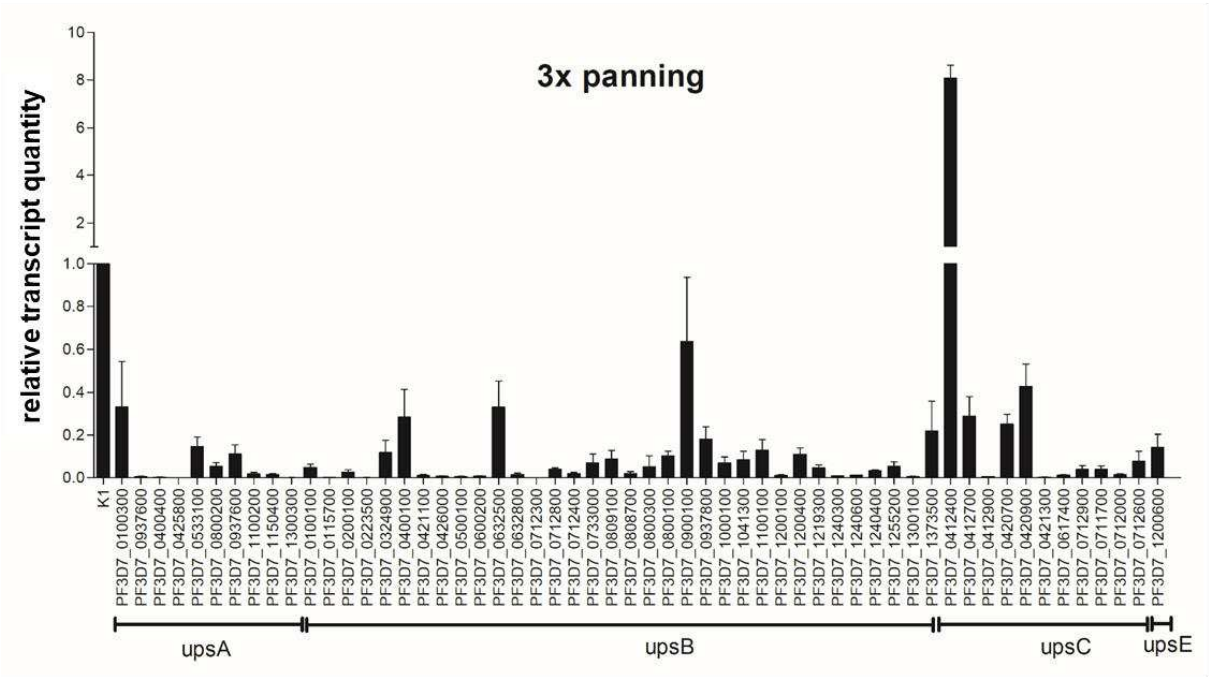
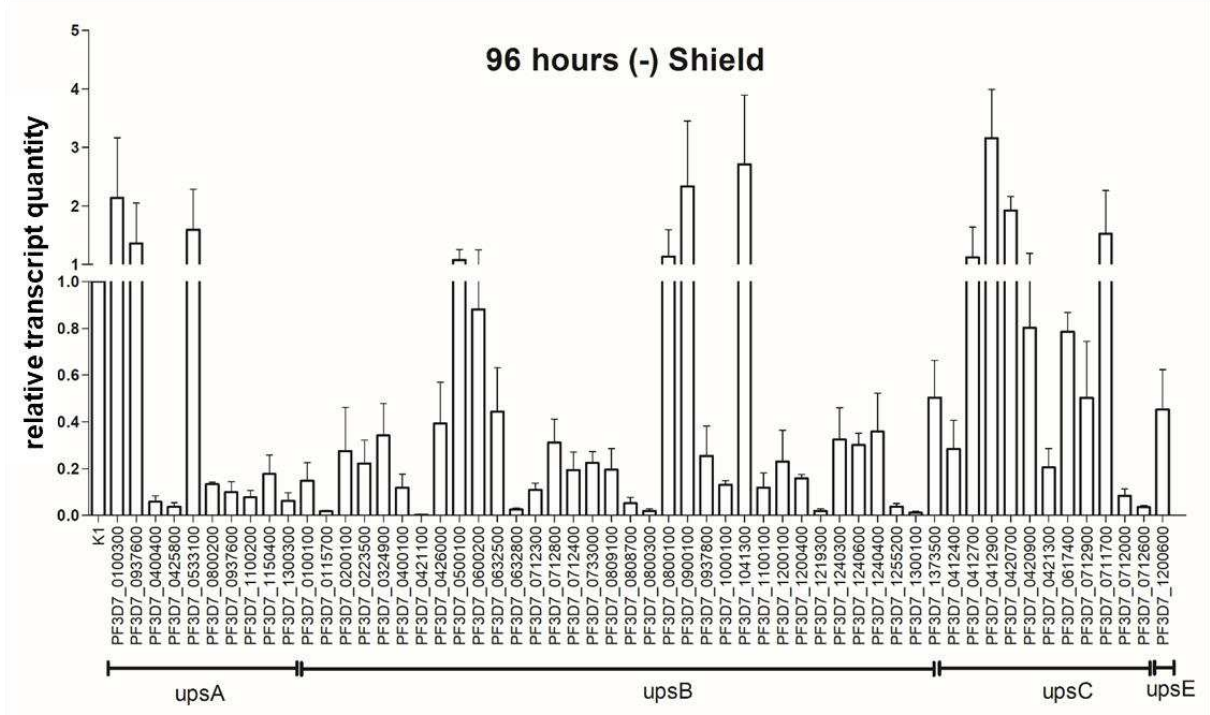
Figure 3

A



B



362

C

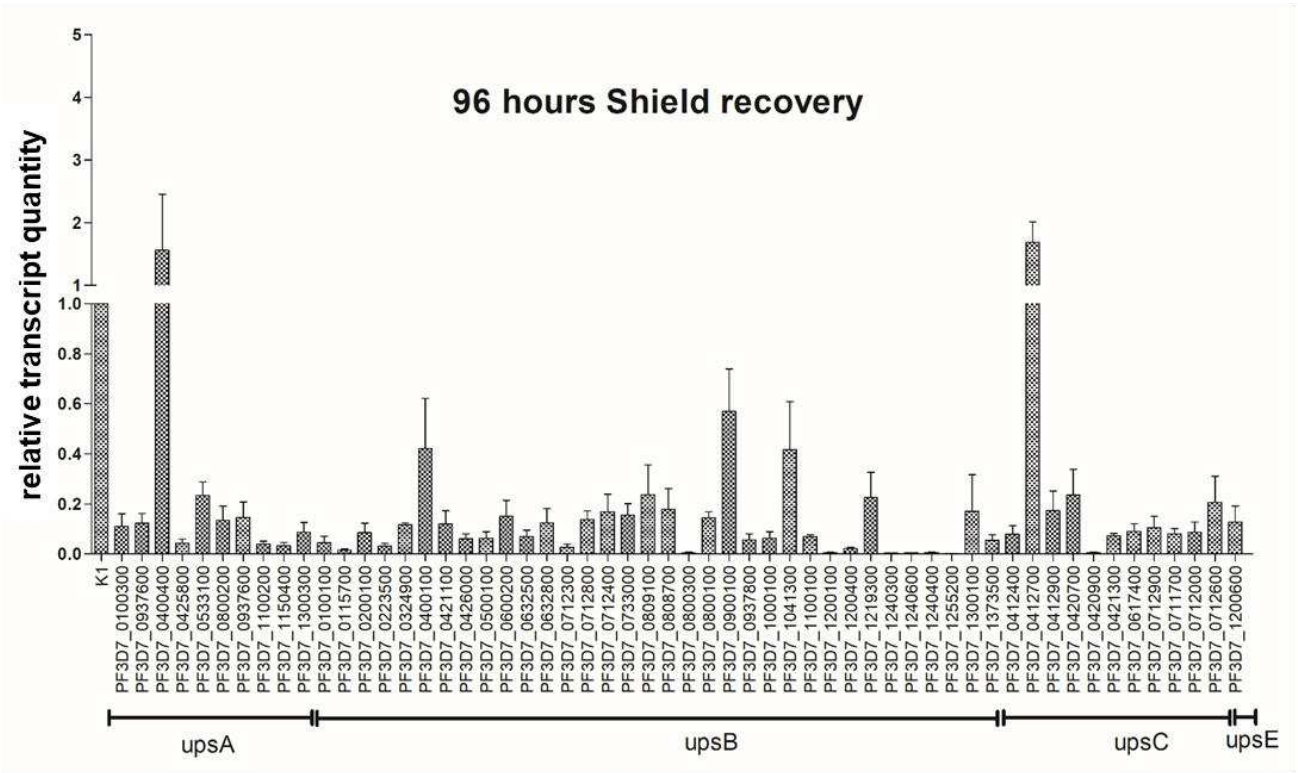


Figure 3: *var* gene transcription analysis during a transient *knockdown* in PfAP2-O-GFP-HA-DD24 parasites. A: RNAs extracted from NF54::PfAP2-O-GFP-DD24 parasites grown in the presence of Shield-1 and panned three times over CHO-CD36 cells were analyzed by RT-qPCR using t-seryl ligase ("K1") as an internal control transcript, as described in Methods. **B:** *var* transcript profile after 96 hours incubation of parasites without Shield-1. **C:** *var* transcript profile in parasites after re-establishment of PfAP2-O for two reinvasions (96 h). The data represent results from three independent experiments and error bars indicate the standard deviation between these.

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Figure 4

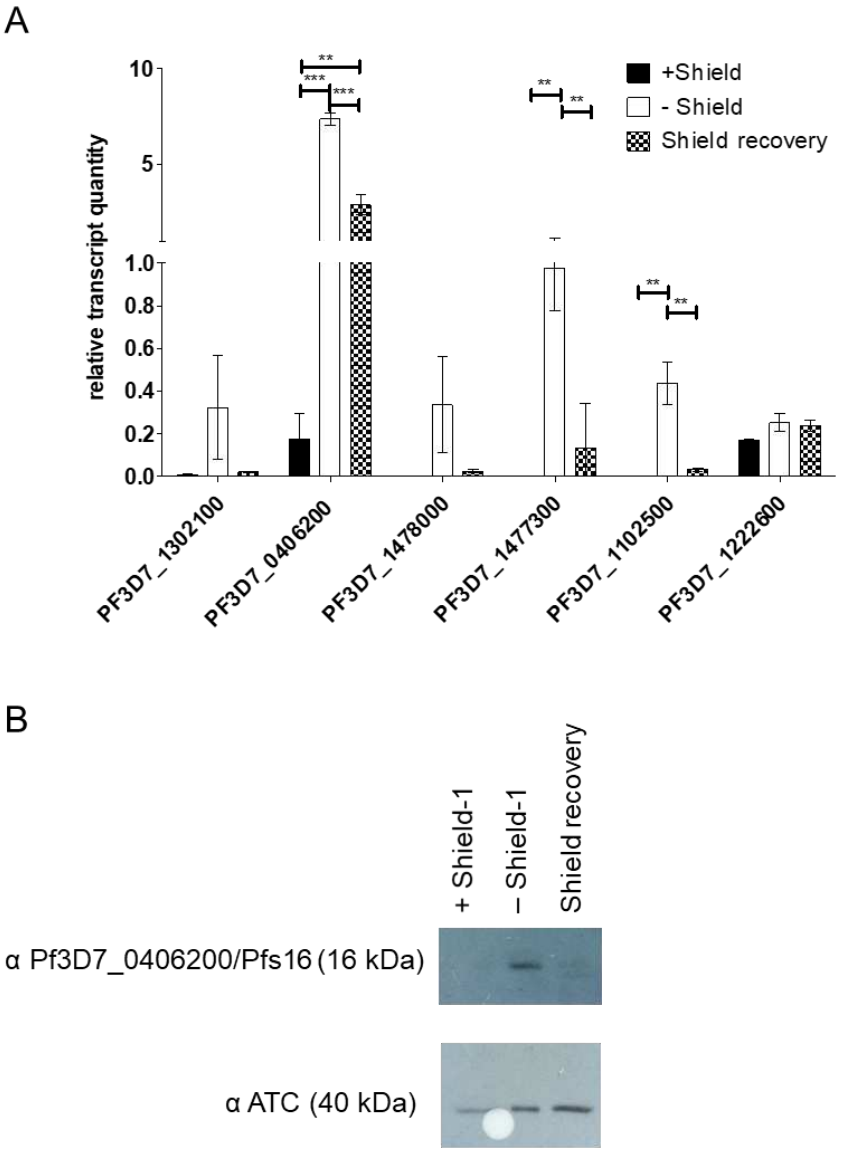
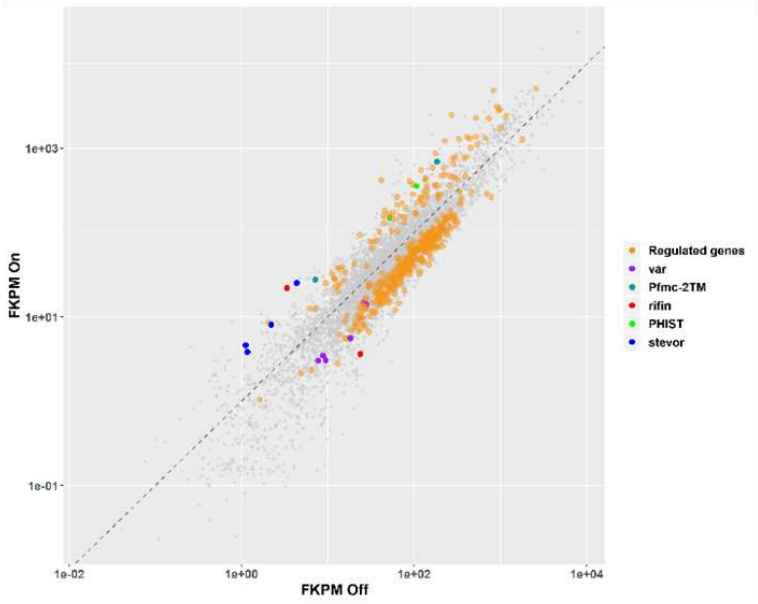


Figure 4: PfAP2-O-GFP-HA-DD24 is involved in the transcription/translation of gametocyte-related genes in *P. falciparum*. A: Transcript abundance of gametocyte-stage specific genes in PfAP2-O-GFP-HA-DD24 expressing, knocked-down (two reinvasions without Shield-1), and recovered parasites (two reinvasion cycles without Shield-1, followed by two reinvasion cycles in the presence of Shield-1). The data were normalized to seryl-tRNA ligase (PF3D7_0913900), used as the internal control. For statistical evaluation, the ANOVA test with Bonferroni's correction was used. " ** " is $p<0.01$ and " *** " is $p<0.005$. **B:** Western blot detecting Pfs16/Pf3D7_0406200 in extracts of NF54::PfAP2-O_GFP_HA_DD24 parasites in the three conditions (on Shield-1/knocked-down/recovered parasites). As a control, an antiATC antibody was used as before.

A

Figure 5



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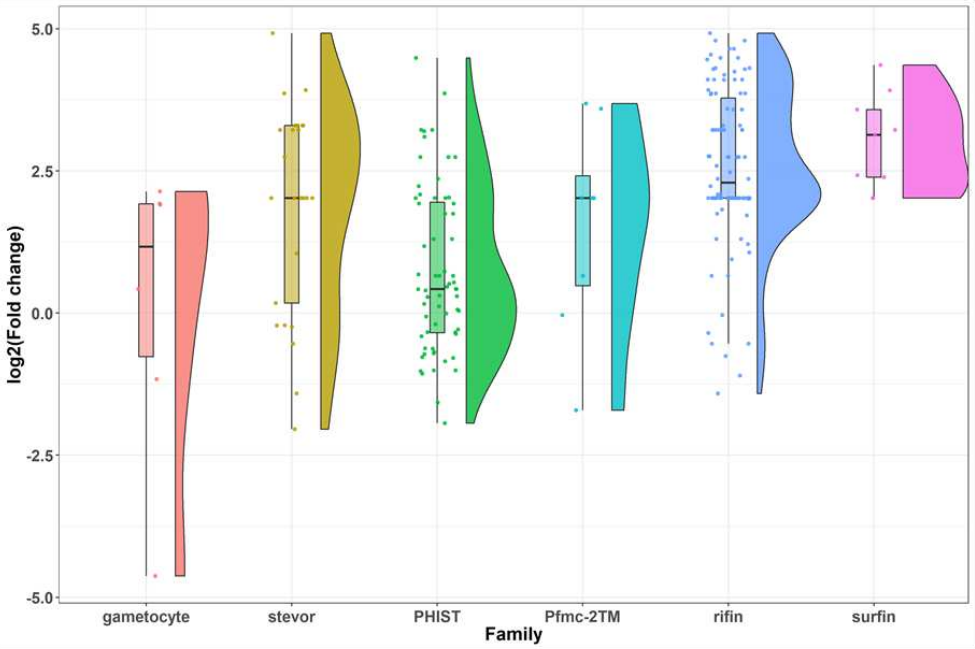

Figure 5: RNAseq analysis in PfAP2-O-GFP-HA-DD24 expressing or knocked down parasites in independent biological triplicates shows influence of PfAP2-O on variant gene transcripts. A: FPKM values in PfAP2-0-GFP-HA-DD24 + Shield-1 parasites "ON" (Y axis) versus PfAP2-O-GFP-HA-DD24 - Shield-1 Parasites "OFF" (X axis). Differentially expressed genes are shown in orange and genes where no significant changes were observed are in grey. Differentially expressed genes of multigenic families are highlighted in colours (Baggerley's test with Bonferroni correction). Data are shown in log10 scale. **B:** Box/Whiskers and violin-plots of RNAseq results comparing indicated transcripts from PfAP2-O knocked-down and expressing parasites. Fold-change is shown in Log2 scale.

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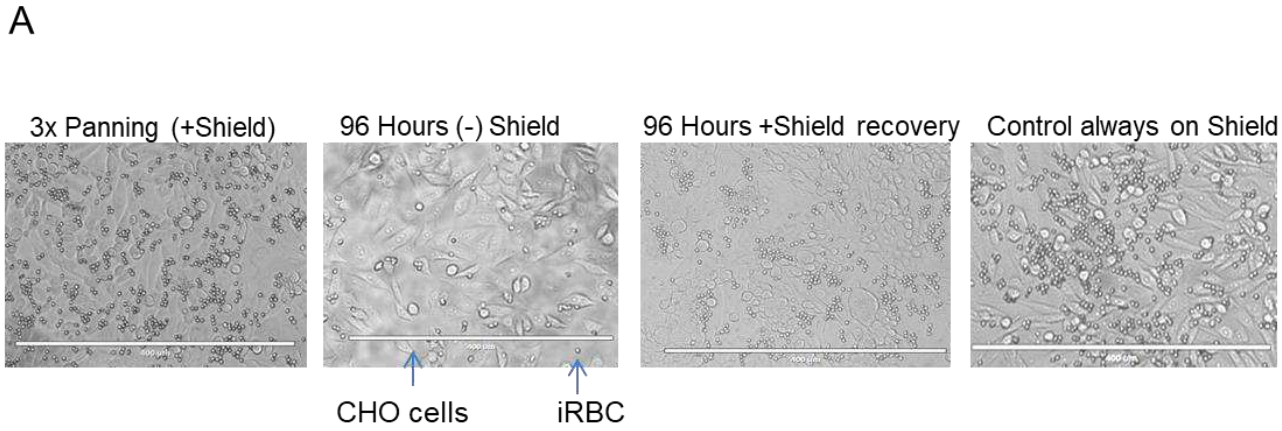
Figure 6



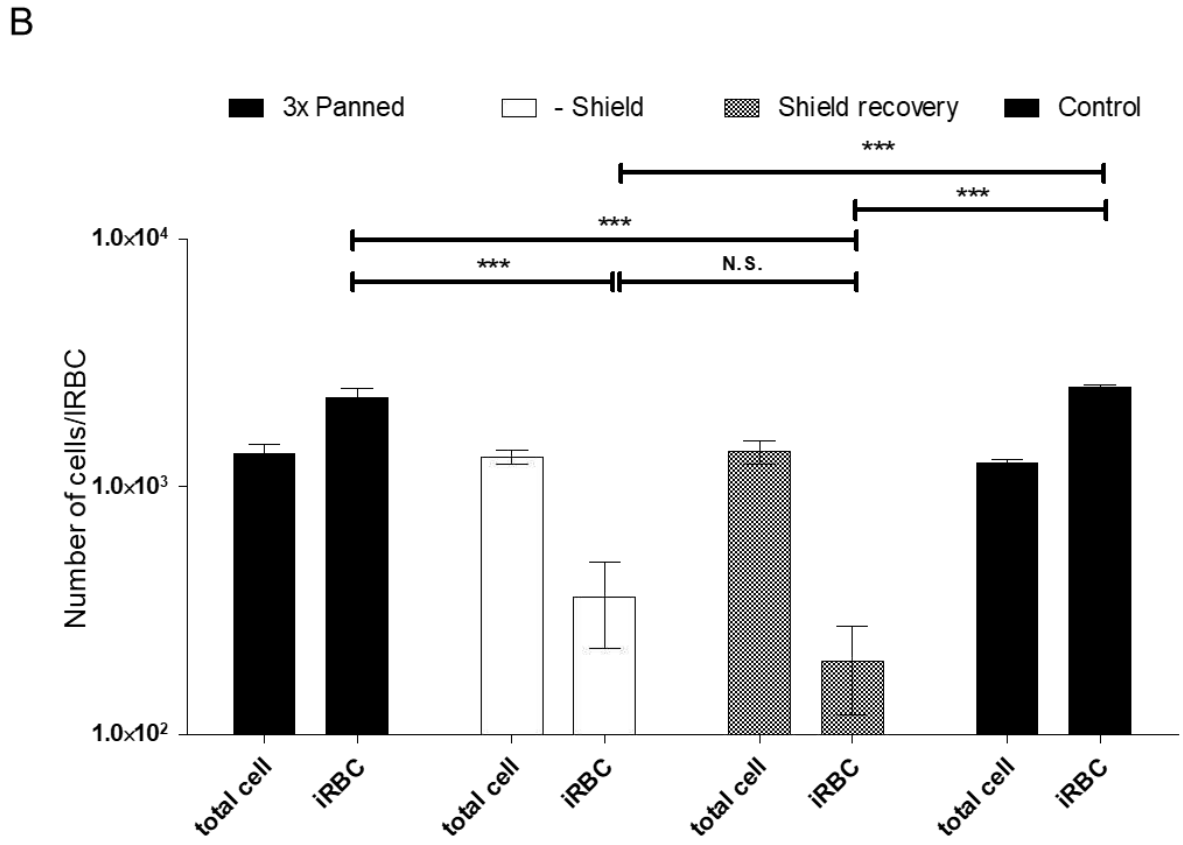
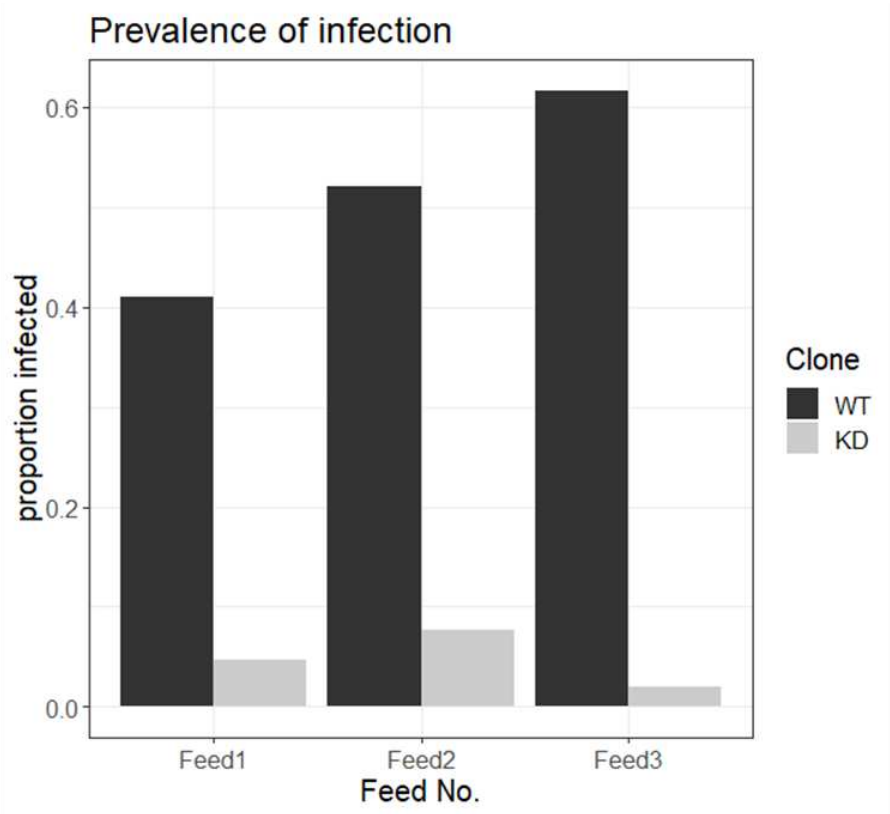


Figure 6. **Adherent phenotype analysis shows decreased cytoadherence of IRBC during and after knockdown**. **A**: Illustrative recording of each condition. The white bar at the bottom of each picture indicates 400 µm. **B**: Graphic representation of three independent experiments. The significance of difference in numbers of adhering iRBC was calculated using Student's T-Test, and three asterisks indicate *p* values < 0.005. N.S. is not significant.

Figure 7

A



B

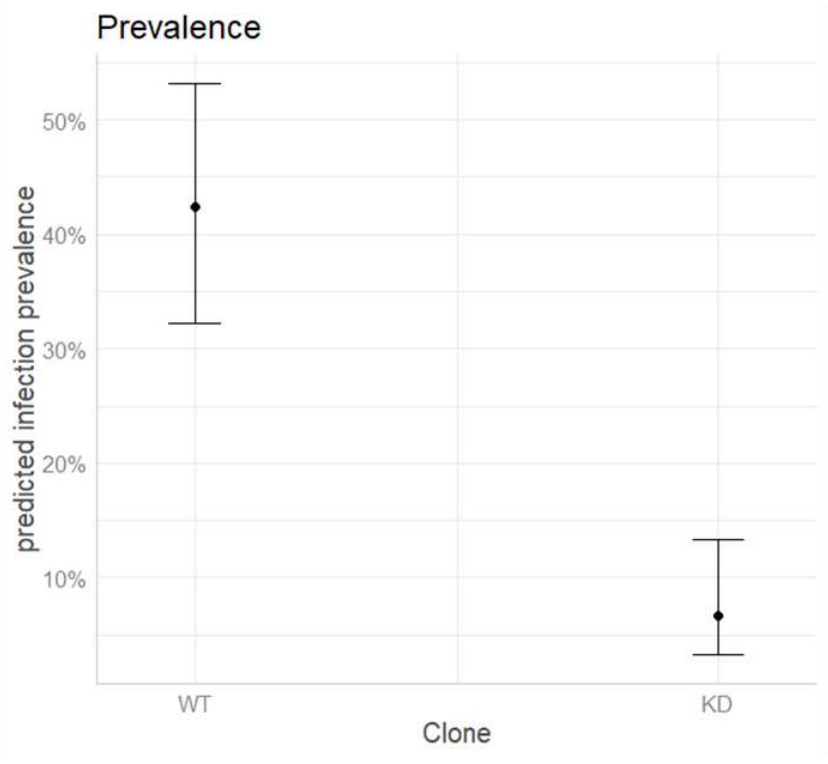


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Figure 7

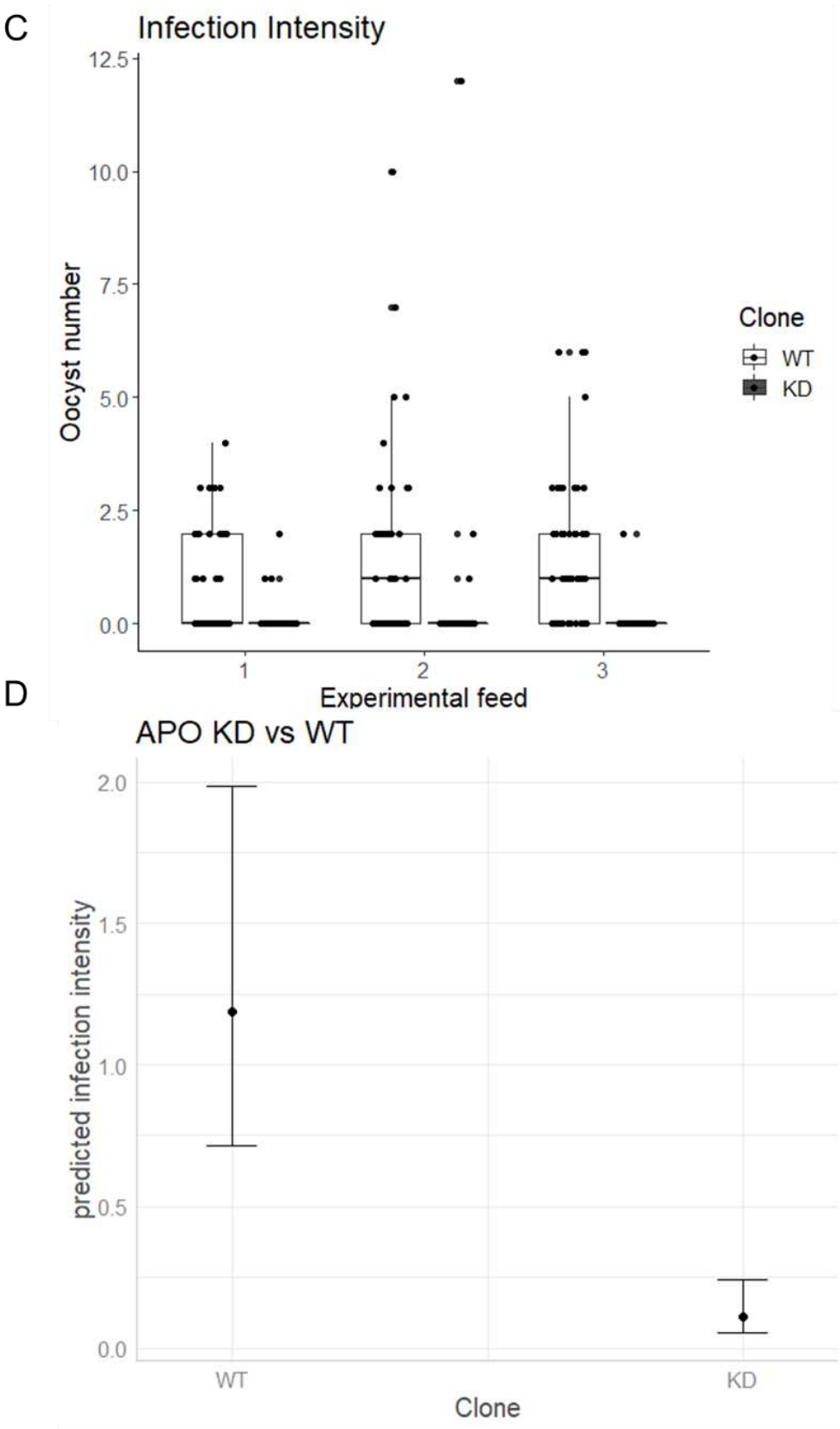

Figure 7. **PfAP2-O is essential for *P. falciparum* transmission to *Anopheles coluzzii***. A. Prevalence of infected mosquitoes fed with ApiAP2-O *knockdown* and WT in each experimental feeding. B. Predicted effect of parasite clone on mosquito infection, bars indicate 95% confidence intervals. C. number of oocysts observed in each experimental feed. D. predicted effect of each clone in oocyst number. Bars indicate 95% confidence intervals.

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Material and Methods

P. falciparum culture

P. falciparum NF54 parasites and transgenic strains were cultured in 4 % hematocrit in RPMI supplemented with 0.25 % Albumax 1 (Invitrogen)/5% human plasma under a 90% N_2, 5% CO_2, 5% O_2 atmosphere. Culture medium was changed daily or every two days and parasites were supplemented with fresh blood every 4 days or more often depending on the parasitemia in the culture. The strains containing a destabilizing domain were maintained in the presence of Shield-1 at 0.5 µM that stabilizes the target protein [27].

In order to obtain synchronized cultures, mature stage parasites were frequently (every 3 weeks) floated using Voluven 6% (Fresenius Kabi) using the method published by Lelievre and colleagues [52]. When purified ring stages were required, cultures with predominantly this form (up to 8 h after reinvasion) were treated for 10 min with 20 volumes of 5% sorbitol solution at room temperature [53].

Molecular cloning/Plasmid construction

P. falciparum knock-in expression plasmids were constructed using the PfAP2-O 3'-end portion. This region was cloned into the transfection plasmid p_GFP_HA_DD24 (figure 1A). First, genomic DNA (gDNA) was prepared from NF54 parasites using the Proteinase K/Phenol-Chloroform method described in Methods in Malaria Research [54]. The final DNA pellet was resuspended in TE and used as a template for PCR using oligonucleotide primers PfAP2-O fw: 5'-AGATCTGAATTGTTCAGAACATTTAAATATAGTACC and PfAP2-O rev: 5'-CTGCAGTAAATTATTAAGGGGGATGTTATTATTAAC (restriction sites BglII and PstI underlined).

The oligonucleotides were used in standard PCRs (30 cycles of 94°C, 40 s, 50°C for 40 s and 65°C for 90 s, final polymerization of 10 min at 65°C). The PCR products were excised and purified via the glassmilk method [55], ligated into pGEM T easy (Promega) and transformed in DH10B chemically competent *E. coli* cells. After plating and growth, colonies were grown in Terrific Broth - ampicillin (TB-amp) supplemented liquid medium and the plasmids extracted using a standard miniprep protocol [56]. Clones were sequenced by the dideoxy-dNTP method and checked for their integrity. The correct fragment was subcloned in the p_GFP_HA_DD24 vector via PstI/BglII. Recombinant plasmids used for transfection were retransformed in *E. coli* SURE cells and grown in 200 ml TB-amp cultures of which plasmids were recovered by the maxiprep protocol [56].

Transfection of blood stage parasites

Empty erythrocytes were electroporated with 40 µg of maxiprep-purified plasmid in incomplete cytomix following the Hasenkamp protocol [57] with slight modifications. $2*10^7$ mature parasites (schizonts) were concentrated up to 80% parasitemia and mixed with the electroporated

erythrocytes. After transfection, culture media were changed daily and parasites were submitted to drug pressure with 2.5 nM WR99210 (a gift from Jacobus Inc, USA) 48 h after transfection. Transfected parasites had their medium changed daily until day 6 when no more live parasites were visible. Once parasites reappeared (normally 16-25 days after transfection) and ring stage parasitemia reached 3-4 %, several aliquots were frozen as backups. WR99210-resistant parasites were then cultivated for 20 days without WR99210 to promote the loss of episomal plasmids in parasites that did not recombine in the target locus. Then, WR99210 was re-added until parasitemia increased again and new aliquots were frozen. These drug on-off cycles were repeated for three times in order to enrich recombinant parasites. Then, parasites were cloned by limiting dilution and DNA was analyzed in order to identify clonal populations with the PfAP2-O locus modified using the oligonucleotides PfAP2-O "check" 5'-CATCAAAATGGATTTAATAATTGTTC and HA rev 5'-AGCGGCATAATCTGGAACATCGTAC.

RNA purification and cDNA synthesis

Total RNA was converted to cDNA following the protocol described in [28]. When parasitemias reached 5%, RNA from parasites with and without Shield-1 treatment were harvested using Trizol (Life Technologies) following the manufacturer's instructions. The final RNA pellet resuspended in nuclease free water. For reverse transcription, 10 µg of total RNA were digested three consecutive times by the enzyme DNAse I (Fermentas). The material obtained from this reaction was used for cDNA synthesis using RevertAid reverse transcriptase (Fermentas) using random hexamer oligonucleotides (N_6). Relative transcript quantities of different genes (see a list of oligos used for RT-PCR in Supplementary Table 5) were then calculated by the $2^{-\Delta Ct}$ method [58] using the seryl tRNA ligase transcript as endogenous control.

RNAseq with parasite-derived cDNA

Total RNA was isolated from the harvested ring-stage parasites with Trizol reagent as described above. The concentration of the isolated RNA was quantified using a Nanodrop device (Thermo Scientific, USA) and the quality of the RNA was measured by a 2100 Bioanalyzer (Agilent Technologies, CA). Then, the TruSeq RNA Sample Preparation Kit v2 low sample (LS) protocol (Illumina Inc., CA), was used to construct cDNA libraries from 9 samples: 3 independent samples with Shield-1 (control), 3 independent samples without Shield-1 (knock down), and 3 independent samples after Shield-1 re-addition (recovery). RNAseq was conducted in an Illumina NextSeq500 sequencer following the recommendations of the provider using mid output flow cells and a total of 10 million reads per sample. The CLC Genomics Workbench 7.01 program was used to remove the adapter and assess read quality from the raw reads. The same platform was used to subject reads to the reference *P. falciparum* genome available in the Ensemble Genome database (Release 26) and to generate gene read count tables and FKPM values as well as to calculate the fold change of each gene (Supplementary Table 2).

443 The data visualization plots were obtained using R statistical software version 3.5.5 using the tidyverse
444 package version 1.2.1 [59].

445 ## Pull down of proteins associated with PF3D7_1143100_GFP_HA_DD24

446 A total protein extract from the pPF3D7_1143100_GFP_HA_DD24 clone was prepared and the
447 protein complex containing the HA tag was purified using the HA-Tag (C29F4) Rabbit mAb (Sepharose
448 Beads conjugate, Cell Signaling Technology) kit. The obtained eluate was separated in a 12% SDS gel
449 using immunoprecipitated NF54 wild-type parasites as a control. To detect proteins in the precipitated
450 complex, the SDS gel was submitted to silver staining using the PlusOne Silver Staining Kit (GE
451 Healthcare, Supplementary figure 3). We observed three different protein bands in the extract from
452 the modified parasites compared to the negative control. These bands and bands corresponding to the
453 same size in the negative control were excised, trypsin-digested and analyzed by mass spectrometry.
454 Analyses were performed on LTQ-Orbitrap Velos ETD (Thermo) coupled with Easy nanoLC II (Thermo).
455 The peptides were separated on a C18RP column on a 95 min gradient. The instrumental conditions
456 were checked using 50 fmol of a tryptic digest of BSA as standard. The sample carryover was
457 completely removed between runs. The peptide search was conducted against the protein database
458 *Plasmodium falciparum*_reviewed Uniprot.

459 ## Adherent phenotype selection of blood stage parasites in static assays (panning)

460 The selection of adhesive phenotypes of infected red blood cells (IRBC) adherent to CHO-CD36
461 was done essentially as described in Gölnitz and colleagues [28]. Briefly, Voluven-floated trophozoite
462 stage IRBC (5 x10^7-10^8) were layered over confluent CHO-CD36 cells in RPMI/human plasma at pH6.8.
463 The IRBC were incubated over the cells for one hour at 37°C and gently mixed every 15 min. After 1 h,
464 the non-adherent IRBC were aspired and the remaining adherent IRBC were washed three times with
465 RPMI at pH 6.8 and after the last washing step the number of adherent IRBC was documented. The
466 remaining IRBC were then detached using RPMI/plasma at pH 7.2-7.4 and returned to the normal
467 culture conditions. The process was repeated 3 times after which strongly adherent IRBC
468 predominantly expressing *var* gene PF3D7_0412400 (CHO-CD36 binding) were obtained. For the
469 silencing assay, cells were grown in 6well plates and central areas were marked in each well. For the
470 cytoadherence readout, ten pictures for each condition were taken using the EVOS FL digital inverted
471 microscope (AMG). The CHO cells and parasites were counted and the results were plotted using
472 GraphPad Prism 5 software.
473

474 ## Fluorescence Microscopy

475 For GFP expression analysis, parasites were fixed as described in [60] followed by incubation with
476 PBS/Saponin 0.01% and DAPI at a final concentration of 2 µg/mL at 37°C for 1 h. After that, the

parasites were washed 3 times with PBS/Saponin 0.01% and incubated with the membrane marker Wheat Germ agglutinin (WGA) Texas Red™-X conjugate (Invitrogen) in PBS/Saponin 0.01% for 20 minutes at 37°C. Finally, parasites were washed three times with PBS/Saponin. Images were acquired in a fluorescence microscope Zeiss Axio Observer Z1 and processed using Photoshop version 5.

Western blot

Total protein extract was obtained from saponin-lysed parasites, separated in 10% SDS polyacrylamide gels and transferred to a Hybond C nitrocellulose membrane (GE Healthcare). After blocking with 4% nonfat milk in 1xPBS/0.1% Tween20, membranes were incubated with a murine antiHA antibody (Sigma-Aldrich, 1:1000) or anti-Pfs16 followed by an antiMouse IgG-peroxydase antibody (KPL, 1:5000) incubation. Blots were washed with PBS/Tween between incubations and finally incubated with WesternPico Super signal substrate (Pierce/Thermo) for detection. As a loading control, a murine polyclonal antiATC antibody (1:1000) was used. The signal intensities were measured using the ImageJ program (NIH).

Mosquito infection with *P. falciparum* gametocytes

Plasmodium falciparum wild type (NF54) and transgenic parasites were maintained under standard conditions to produce infectious gametocytes as described [61] in the absence of Shield-1. For each parasite line, 100 mosquitoes of *An. coluzzii* Ngousso strain were infected using membrane feeding [62], unfed mosquitoes were removed and the blood fed mosquitoes were maintained under standard insectary conditions (26 ± 1 °C, 80% humidity, 12 h light:12 h dark cycle) and fed ad libitum on 5% glucose solution containing 0.05% (w/v) 4-aminobenzoic acid (PABA). Mosquitoes were dissected 10 days post-infection and the midguts were examined microscopically (400x magnification) for the presence and number of oocysts. Permission for the non-therapeutic use of human blood was obtained from the Scottish National Blood Transfusion Service Committee for the Governance of Blood and Tissue Samples for Non-Therapeutic Use, reference 18-15.

Statistical analysis of mosquito infection

To estimate the effect of the PfApiAP2-Sp and PfApiAP2-O knockout/knockdown in *P. falciparum* transmission, a generalized linear mixed-effect model GLMM was employed using the package lme4 [63] in R software version 3.5.3 [64]. To assess the effect on infection prevalence, a binomial GLMM with log link was used with fixed variables of parasite clone and gametocyte number present in the bloodmeal, with the replicate (infectious feed number) included as a random effect. A stepwise backward selection was used to identify the significant fixed effects [65], with optimal model selection performed using the Akaike Information Criterion (AIC) for which lower scores indicate better model fits, and with likelihood ratio tests were used to compare the different models. Model diagnostics, including normality of residuals were done by visual inspection of residuals versus fitted values for each random effect group,

and a Shapiro-Wilk test. Finally, to calculate how much of the variance was explained by the variables included in the final model, an R squared for the generalized linear mixed effect model r2glmm [66], was applied. For this, the package MuMIn, version 1.41.0 was used, and the R2m indicates the variation in prevalence explained by the fixed effects and the R2c included all the variables used in the model.

For intensity of infection (model 2), a zero-inflated negative binomial distribution was fitted to the data in a GLMM analysis using the package glmmTMB [67], with the family nbinom 2 (log link). The final minimum model included fixed variables of clone and the number of gametocytes present in the bloodmeal, with feed number as a random effect: For model selection, diagnostics and to test overdispersion the same procedures from model 1 were used. As above, R software version 3.5.3 [68] was used for data analysis and models fit, as additional packages were used lme4 [63] , DHARMa 0.3.0 [69] aods3 [70] and MuMIn (v1.42.5) [66].

Acknowledgments

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Literature cited

1. World Health Organization. *World Malaria Report 2019. Geneva.* (2019).

2. Dondorp, A. M. *et al.* Artemisinin Resistance in Plasmodium falciparum Malaria. *n engl j med Mae Sot Fam. Heal. Int. Churchill Hosp. N.J.W. N Engl J Med* **3615361**, 455–67 (2009).

3. WHO | Insecticide resistance. *WHO* (2020).

4. Delves, M. J., Angrisano, F. & Blagborough, A. M. Antimalarial Transmission-Blocking Interventions: Past, Present, and Future. *Trends Parasitol.* **34**, 735–746 (2018).

5. Su, X. Z. *et al.* The large diverse gene family var encodes proteins involved in cytoadherence and antigenic variation of Plasmodium falciparum-infected erythrocytes. *Cell* **82**, 89–100 (1995).

6. Scherf, A. *et al.* Antigenic variation in malaria: in situ switching, relaxed and mutually exclusive transcription of var genes during intra-erythrocytic development in Plasmodium falciparum. *EMBO J* **17**, 5418–5426 (1998).

7. Chen, Q. *et al.* Developmental selection of var gene expression in Plasmodium falciparum. *Nature* **394**, 392–395 (1998).

8. Brancucci, N. M. B., Witmer, K., Schmid, C. D., Flueck, C. & Voss, T. S. Identification of a cis-acting DNA-protein interaction implicated in singular var gene choice in Plasmodium falciparum. *Cell. Microbiol.* **14**, 1836–1848 (2012).

9. Amit-Avraham, I. *et al.* Antisense long noncoding RNAs regulate var gene activation in the malaria parasite Plasmodium falciparum. *Proc. Natl. Acad. Sci. U. S. A.* **112**, 982–991 (2015).

10. Jing, Q. *et al.* Plasmodium falciparum var Gene Is Activated by Its Antisense Long Noncoding RNA. *Front. Microbiol.* **9**, (2018).

11. Voss, T. S. *et al.* A var gene promoter controls allelic exclusion of virulence genes in Plasmodium falciparum malaria. *Nature* **439**, 1004–1008 (2006).

12. Guizetti, J., Barcons-Simon, A. & Scherf, A. Trans-acting GC-rich non-coding RNA at *var* expression site modulates gene counting in malaria parasite. *Nucleic Acids Res.* gkw664 (2016). doi:10.1093/nar/gkw664

13. Barcons-Simon, A., Cordon-Obras, C., Guizetti, J., Bryant, J. M. & Scherf, A. CRISPR Interference of a Clonally Variant GC-Rich Noncoding RNA Family Leads to General Repression of *var* Genes in *Plasmodium falciparum*. *MBio* **11**, (2020).

14. Fan, Y. *et al.* Rrp6 Regulates Heterochromatic Gene Silencing via ncRNA RUF6 Decay in Malaria Parasites. *MBio* **11**, e01110-20 (2020).

15. Brancucci, N. M. B. *et al.* Heterochromatin Protein 1 Secures Survival and Transmission of Malaria Parasites. *Cell Host Microbe* **16**, 165–176 (2014).

16. Pérez-Toledo, K. *et al.* Plasmodium falciparum heterochromatin protein 1 binds to tri-methylated histone 3 lysine 9 and is linked to mutually exclusive expression of var genes. *Nucleic Acids Res.* **37**, 2596–606 (2009).

17. Li, Z. *et al.* DNA helicase RecQ1 regulates mutually exclusive expression of virulence genes in Plasmodium falciparum via heterochromatin alteration. *Proc. Natl. Acad. Sci. U. S. A.* **116**, 3177–3182 (2019).

18. Lavstsen, T., Salanti, A., Jensen, A. T. R., Arnot, D. E. & Theander, T. G. Sub-grouping of Plasmodium falciparum 3D7 var genes based on sequence analysis of coding and non-coding regions. *Malar. J.* **2**, 27 (2003).

19. Zhang, Q. *et al.* Exonuclease-mediated degradation of nascent RNA silences genes linked to severe malaria. *Nature* **513**, 431–5 (2014).

20. Yuda, M., Iwanaga, S., Shigenobu, S., Kato, T. & Kaneko, I. Transcription factor AP2-Sp and its target genes in malarial sporozoites. *Mol. Microbiol.* **75**, 854–63 (2010).

21. Yuda, M. *et al.* Identification of a transcription factor in the mosquito-invasive stage of malaria parasites. *Mol. Microbiol.* **71**, 1402–14 (2009).

22. Kafsack, B. F. C. *et al.* A transcriptional switch underlies commitment to sexual development in malaria parasites. *Nature* **507**, 248–252 (2014).

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

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

25. Flueck, C. *et al.* A major role for the Plasmodium falciparum ApiAP2 protein PfSIP2 in chromosome end biology. *PLoS Pathog.* **6**, e1000784 (2010).

26. Martins, R. M. *et al.* An ApiAP2 member regulates expression of clonally variant genes of the human malaria parasite Plasmodium falciparum. *Sci. Rep.* **7**, 14042 (2017).

27. de Azevedo, M. F. M. F. M. F. *et al.* Systematic Analysis of FKBP Inducible Degradation Domain Tagging Strategies for the Human Malaria Parasite Plasmodium falciparum. *PLoS One* **7**, e40981 (2012).

28. Gölnitz, U., Albrecht, L. & Wunderlich, G. Var transcription profiling of Plasmodium falciparum 3D7: assignment of cytoadherent phenotypes to dominant transcripts. *Malar. J.* **7**, 14 (2008).

29. Lopez-Rubio, J. J. *et al.* 5' flanking region of var genes nucleate histone modification patterns linked to phenotypic inheritance of virulence traits in malaria parasites. *Mol. Microbiol.* **66**, 1296–305 (2007).

30. Yuda, M., Iwanaga, S., Kaneko, I. & Kato, T. Global transcriptional repression: An initial and essential step for Plasmodium sexual development. *Proc. Natl. Acad. Sci. U. S. A.* **112**, 12824–9 (2015).

31. Iwanaga, S., Kaneko, I., Kato, T. & Yuda, M. Identification of an AP2-family Protein That Is Critical for Malaria Liver Stage Development. *PLoS One* **7**, e47557 (2012).

32. Santos, J. M. *et al.* Red Blood Cell Invasion by the Malaria Parasite Is Coordinated by the PfAP2-I Transcription Factor. *Cell Host Microbe* **21**, 731-741.e10 (2017).

33. Tonkin, C. J. *et al.* Sir2 paralogues cooperate to regulate virulence genes and antigenic variation in Plasmodium falciparum. *PLoS Biol.* **7**, e84 (2009).

34. Guizetti, J. & Scherf, A. Silence, activate, poise and switch! Mechanisms of antigenic variation in Plasmodium falciparum. *Cell. Microbiol.* **15**, 718–26 (2013).

35. Epp, C., Li, F., Howitt, C. A., Chookajorn, T. & Deitsch, K. W. Chromatin associated sense and antisense noncoding RNAs are transcribed from the var gene family of virulence genes of the malaria parasite Plasmodium falciparum. *RNA* **15**, 116–27 (2009).

36. Freitas-Junior, L. H. *et al.* Telomeric heterochromatin propagation and histone acetylation control mutually exclusive expression of antigenic variation genes in malaria parasites. in *Cell* **121**, 25–36 (2005).

37. Oehring, S. C. *et al.* Organellar proteomics reveals hundreds of novel nuclear proteins in the malaria parasite Plasmodium falciparum. *Genome Biol.* **13**, R108 (2012).

38. Chookajorn, T. *et al.* Epigenetic memory at malaria virulence genes. in *Proc Natl Acad Sci U S A* **104**, 899–902 (2007).

39. Salcedo-Amaya, A. M. *et al.* Dynamic histone H3 epigenome marking during the intraerythrocytic cycle of Plasmodium falciparum. *Proc Natl Acad Sci U S A* **106**, 9655–9660 (2009).

40. Andrews, K. T., Adams, Y., Viebig, N. K., Lanzer, M. & Schwartz-Albiez, R. Adherence of Plasmodium falciparum infected erythrocytes to CHO-745 cells and inhibition of binding by protein A in the presence of human serum. *Int. J. Parasitol.* **35**, 1127–34 (2005).

41. Hsieh, F.-L. *et al.* The structural basis for CD36 binding by the malaria parasite. *Nat. Commun.* **7**, 12837 (2016).

42. Lavstsen, T. *et al.* PNAS Plus: Plasmodium falciparum erythrocyte membrane protein 1 domain cassettes 8 and 13 are associated with severe malaria in children. *Proc. Natl. Acad. Sci.* **109**, 1791–1800 (2012).

43. Warimwe, G. M. *et al.* Plasmodium falciparum var gene expression is modified by host immunity. *Proc. Natl. Acad. Sci. U. S. A.* **106**, 21801–6 (2009).

44. Lavstsen, T. *et al.* Expression of Plasmodium falciparum erythrocyte membrane protein 1 in experimentally infected humans. *Malar. J.* **4**, 21 (2005).

45. Mustaffa, K. M. F., Storm, J., Whittaker, M., Szestak, T. & Craig, A. G. In vitro inhibition and reversal of Plasmodium falciparum cytoadherence to endothelium by monoclonal antibodies to ICAM-1 and CD36. *Malar. J.* **16**, 279 (2017).

46. Jiang, L. *et al.* PfSETvs methylation of histone H3K36 represses virulence genes in Plasmodium falciparum. *Nature* **499**, 223–7 (2013).

47. Dechering, K. J., Thompson, J., Dodemont, H. J., Eling, W. & Konings, R. N. . Developmentally regulated expression of pfs16, a marker for sexual differentiation of the human malaria parasite Plasmodium falciparum. *Mol. Biochem. Parasitol.* **89**, 235–244 (1997).

48. Mair, G. R. *et al.* Regulation of sexual development of Plasmodium by translational repression. *Science (80-.).* **313**, 667–9 (2006).

49. Sinha, A. *et al.* A cascade of DNA-binding proteins for sexual commitment and development in Plasmodium. *Nature* **507**, 253–7 (2014).

50. Filarsky, M. *et al.* GDV1 induces sexual commitment of malaria parasites by antagonizing HP1-dependent gene silencing. *Science (80-.).* **359**, 1259–1263 (2018).

51. Modrzynska, K. *et al.* A Knockout Screen of ApiAP2 Genes Reveals Networks of Interacting Transcriptional Regulators Controlling the Plasmodium Life Cycle. *Cell Host Microbe* **21**, 11–22 (2017).

52. Lelièvre, J. *et al.* An alternative method for Plasmodium culture synchronization. *Exp Parasitol* **109**, 195–197 (2005).

53. Lambros, C. & Vanderberg, J. P. Synchronization of Plasmodium falciparum erythrocytic stages in culture. *J Parasitol* **65**, 418–420 (1979).

54. Ljungström, I., Perlmann, H., Schlichtherle, M., Scherf, A. & Wahlgren, M. *Methods in Malaria Research*. (MR4/ATCC, BioMalPar, 2008).

55. Boyle, J. S. & Lew, A. M. An inexpensive alternative to glassmilk for DNA purification. *Trends Genet* **11**, 8 (1995).

56. Sambrook, J., G. M. *[Molecular Cloning: A Laboratory Manual. 4 ed*. *Cold Spring Harbor Laboratory Press* **1**, (2012).

57. Hasenkamp, S., Russell, K. T. & Horrocks, P. Comparison of the absolute and relative efficiencies of electroporation-based transfection protocols for Plasmodium falciparum. *Malar. J.* **11**, 210 (2012).

58. Livak, K. J. & Schmittgen, T. D. Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2−ΔΔCT Method. *Methods* **25**, 402–408 (2001).

59. Wickham, H. tidyverse: Easily Install and Load the 'Tidyverse'. R package version 1.2.1. https://CRAN.R-project.org/package=tidyverse. (2017).

60. Tonkin, C. J. *et al.* Localization of organellar proteins in Plasmodium falciparum using a novel set of transfection vectors and a new immunofluorescence fixation method. *Mol. Biochem. Parasitol.* **137**, 13–21 (2004).

61. Hyde, J. E., Carter, R., Ranford-Cartwright, L. & Alano, P. The Culture and Preparation of Gametocytes of Plasmodium falciparum for Immunochemical, Molecular, and Mosquito Infectivity Studies. *Protoc. Mol. Parasitol.* **27**, 67–88 (2003).

62. Ranford-Cartwright, L. C. *et al.* Characterisation of Species and Diversity of Anopheles gambiae Keele Colony. *PLoS One* **11**, e0168999 (2016).

63. Bates, D., Mächler, M., Bolker, B. M. & Walker, S. C. Fitting linear mixed-effects models using lme4. *J. Stat. Softw.* **67**, 1–48 (2015).

64. Team, R. C. R: A Language and Environment for Statistical Computing. (2013).

65. Burnham, K. & Anderson, D. *Model Selection and Multimodel Inference: A Practical Information-Theoretic Approach*. (Springer Verlag, 2002).

66. Nakagawa, S., Johnson, P. C. D. & Schielzeth, H. The coefficient of determination R^2 and intra-class correlation coefficient from generalized linear mixed-effects models revisited and expanded. *J. R. Soc. Interface* **14**, 20170213 (2017).

67. Brooks, M. E. *et al.* glmmTMB balances speed and flexibility among packages for zero-inflated generalized linear mixed modeling. *R J.* **9**, 378–400 (2017).

68. R: The R Project for Statistical Computing.

69. DHARMa: residual diagnostics for hierarchical (multi-level/mixed) regression models.

687 70. Lesnoff, M., Fr>, <matthieu Lesnoff@cirad, Lancelot, R. & Lancelot, M. R. *Package 'aods3' Title*
688 *Analysis of Overdispersed Data using S3 Methods*. (2018).

689 ## Supplementary figure

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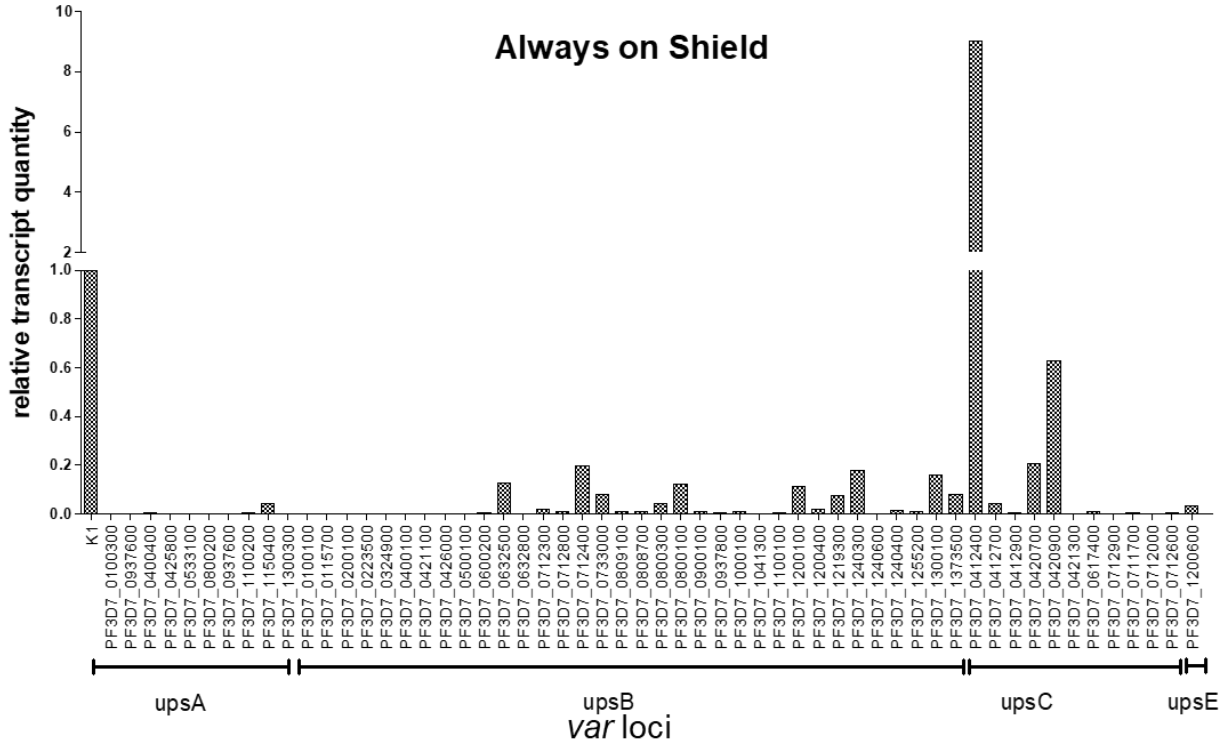

Supplementary figure 1: *var* transcription profile in parasites never submitted to silencing of PfAP2-O (Always on Shield-1, at the end of the knockdown experiment), measured by RT-qPCR as described. K1 is the transcript of t-seryl ligase, used as an internal control (see Methods for details).

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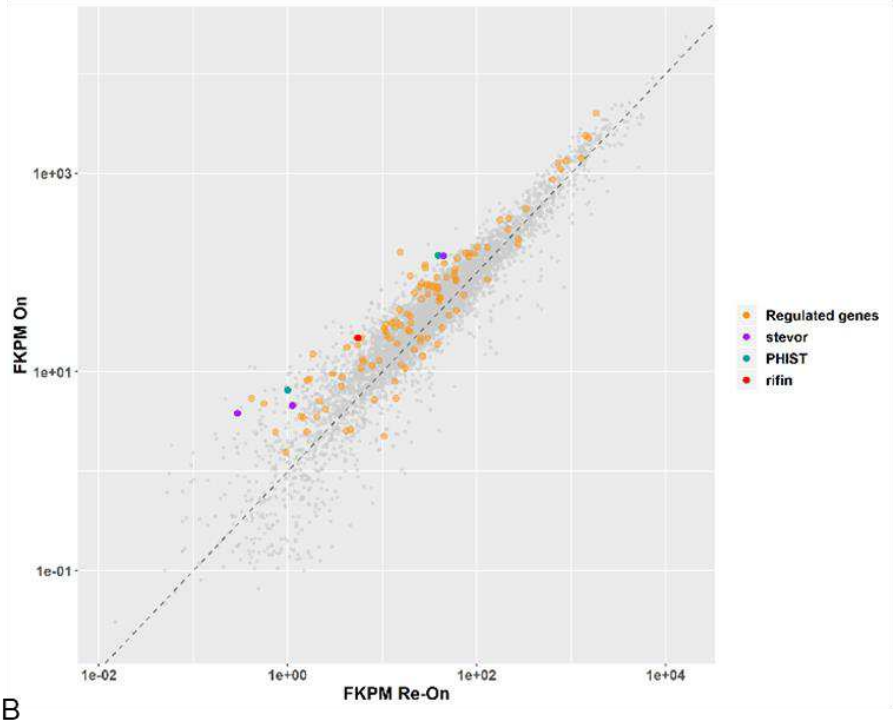
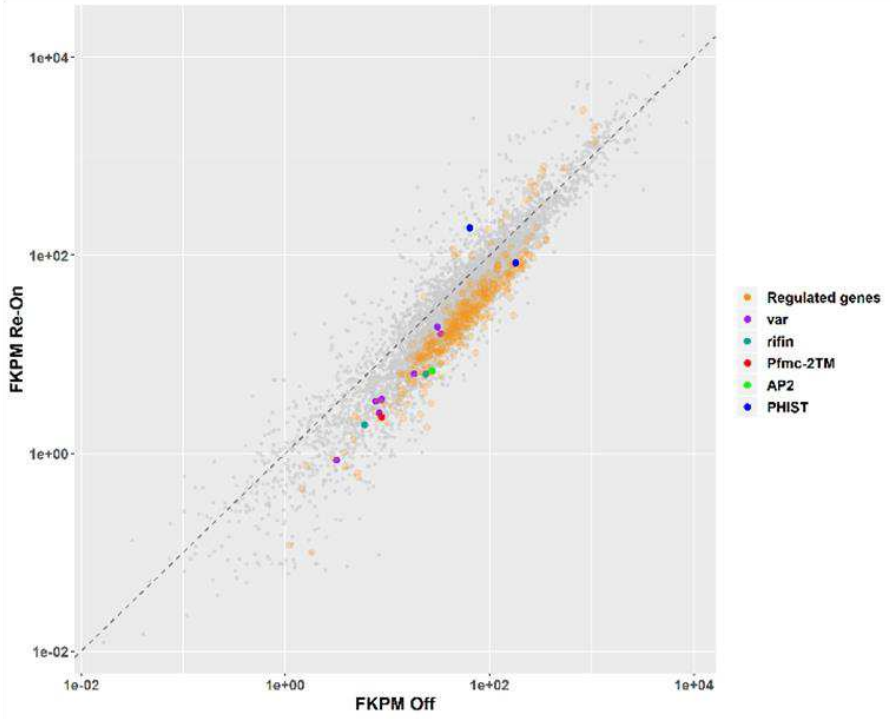
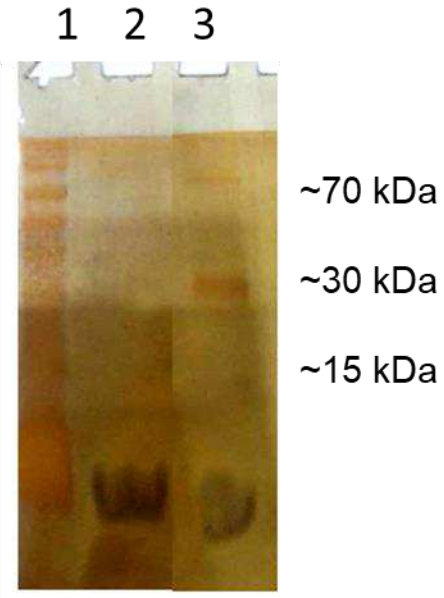
A



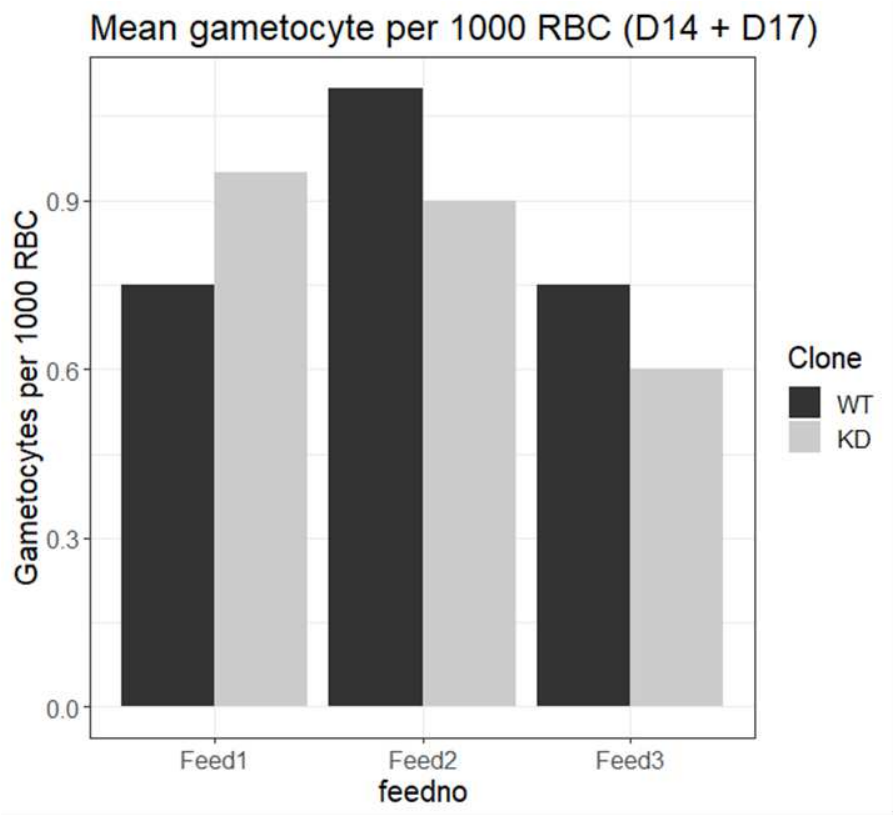
B



Supplementary figure 2. RNA seq analysis in PfAP2-O expressing and knocked-down parasites in biological triplicates **A**: FPKM values in PfAP2-0 + Shield parasites "ON"(Y axis) and PfAP2-O (-) Shield Parasites "OFF" (X axis). The differentially expressed genes are in orange and genes were no statistically significant changes were observed are in grey. Differentially expressed genes of multigenic families are highlighted in colours (Baggerley's test with Bonferroni correction).



Supplementary figure 3. Silver staining of purified protein complexes after antiHA immunoprecipitation. Bands in the size of 70 kDa, 30 kDa and 15 kDa were excised from Coomassie colloidal blue stained gels run in parallel and analyzed by mass spectrometry. Lane 1 represents the molecular weight, lane 2 is the immunoprecipitated NF54 wild type extract and lane 3 the immunoprecipitated protein extract from the PfAP2-O_GFP_HA_DD24 strain. See Methods for details.



Supplementary figure 4. Mean gametocytes number per 1000 RBC counted for each clone in three independent experiment for *Anopheles coluzzzi* infection.

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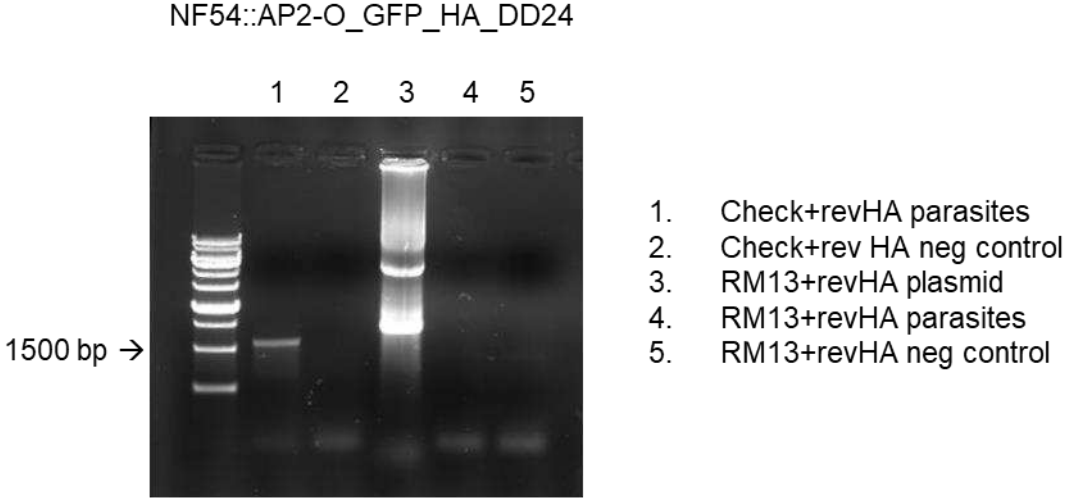
Original pictures/X ray films from Figures 1 and 4



Supplementary figure 4. Original Agarose gel image from the PCR reaction of Figure 1, showing integration of the plasmid in the Pf3D7_1143100 locus.

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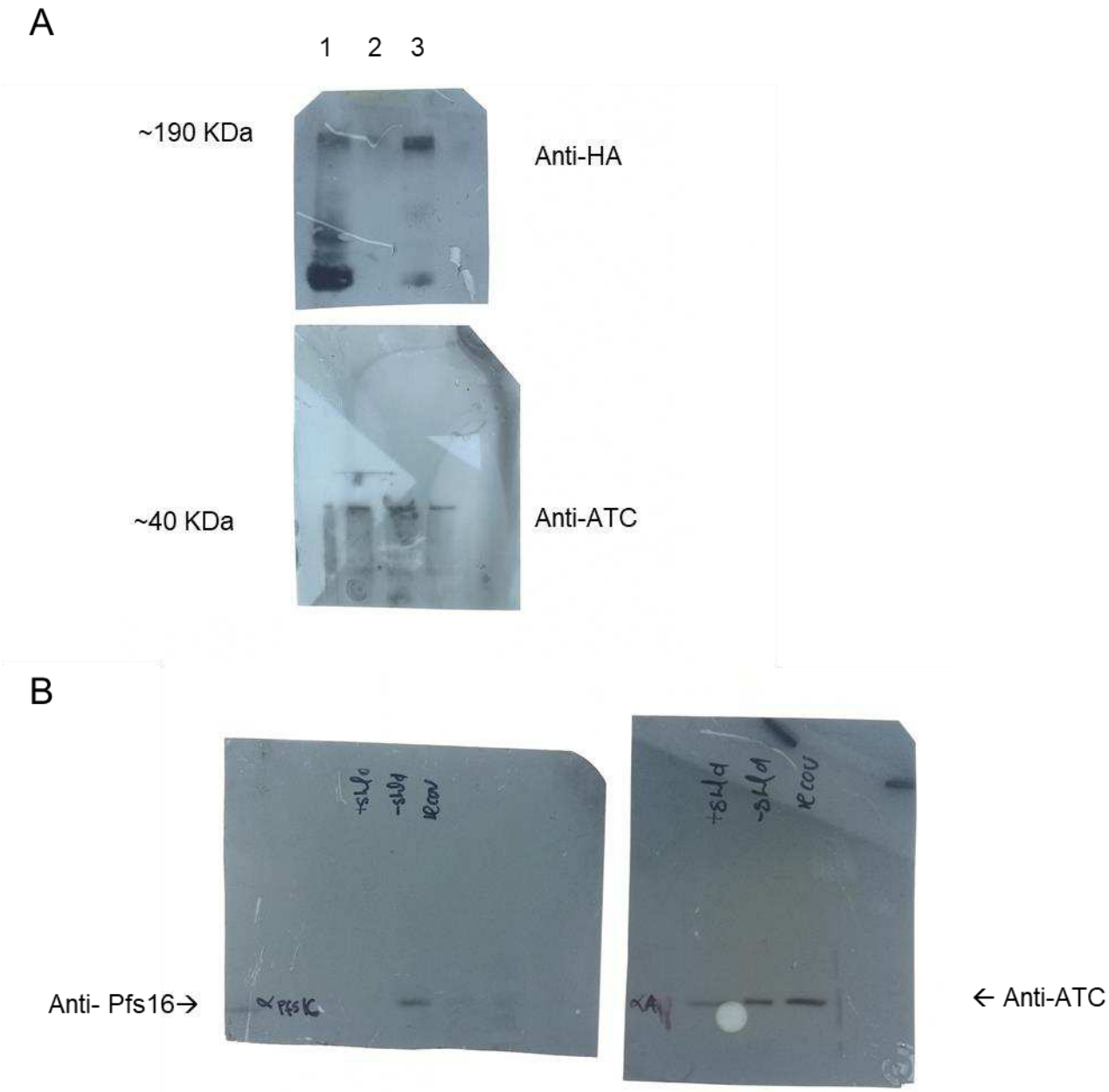

Supplementary figure 5. Original images from western blot results shown in figure 1 and 4. **A:** Original Western blot from figure 1C. Lane 1: Parasites grown in the presence of Shield-1, Lane 2: Parasites grown for two reinvasions without Shield-1, lane 3: Parasites after reestablishment of PfAP2-O-GFP-DD24 by re-addition of Shield-1 for two reinvasion cycles.
B: Original western blot from Figure 4B. Left: Anti Pfs 16. Right: Anti-ATC. See Methods for details.

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Figures

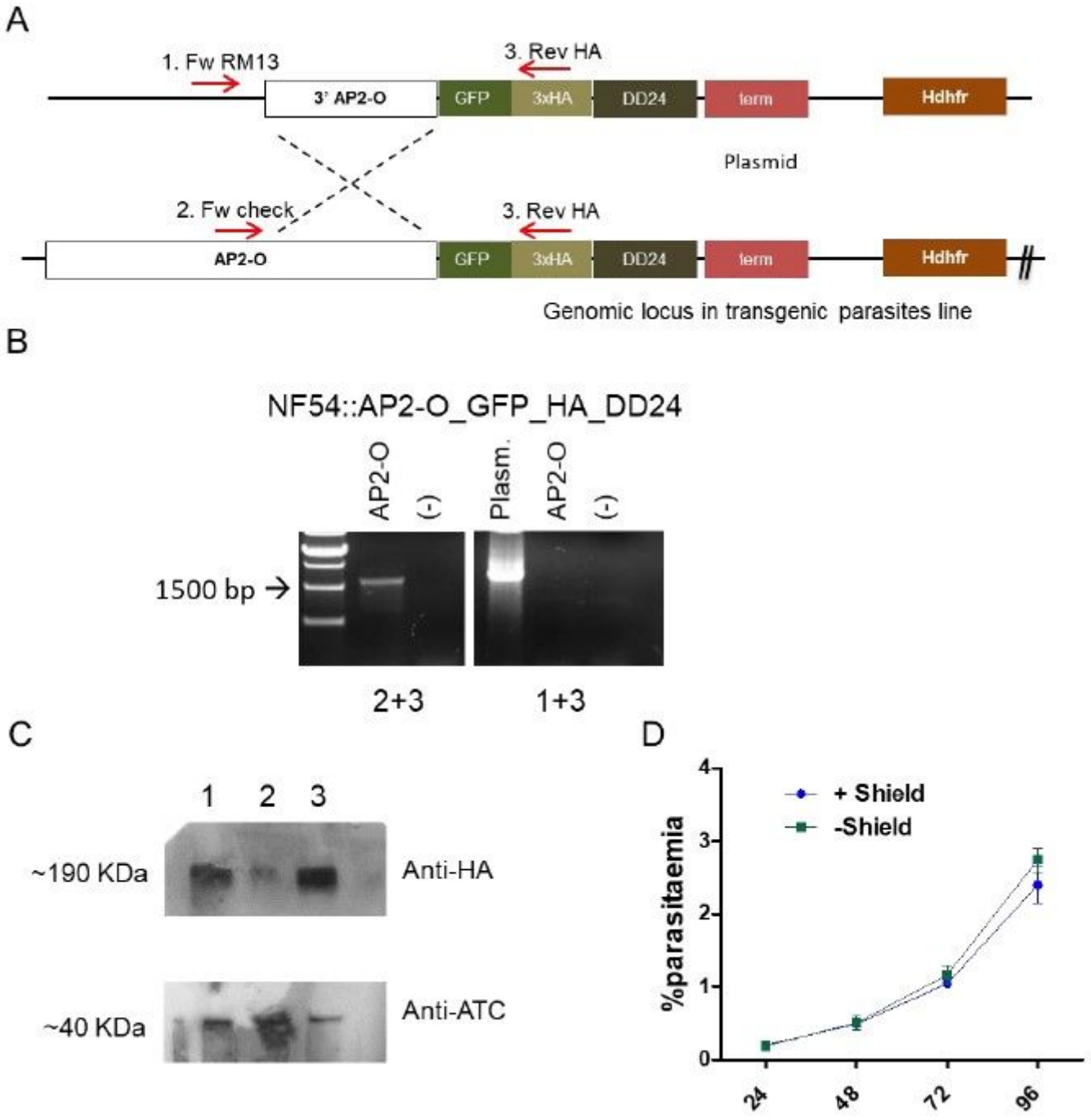

Figure 1

PfAP2-O modification and subsequent knockdown leads to no discernible modification

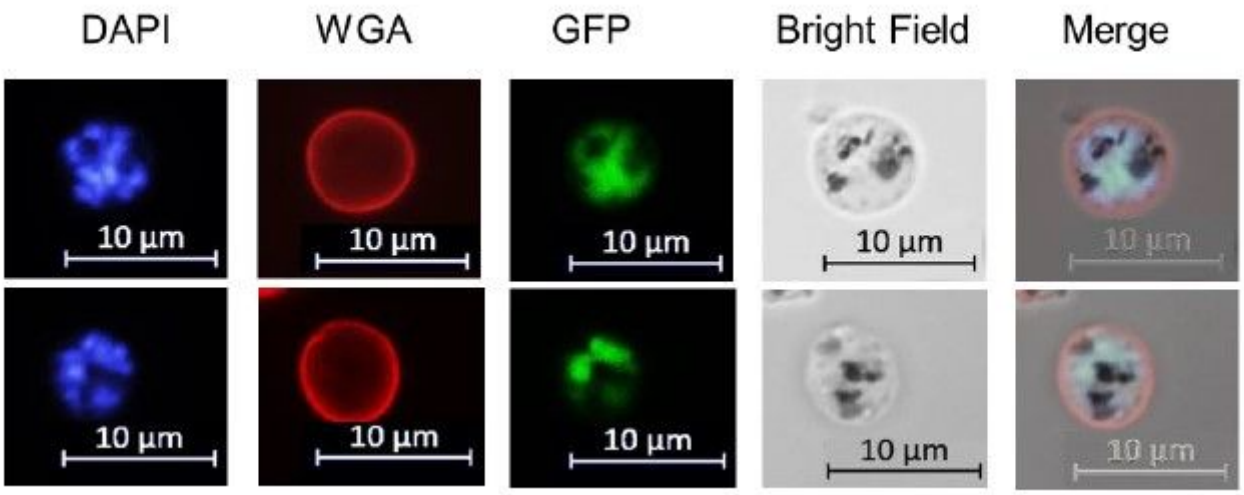

Figure 2

PfAP2-O-GFP-HA-DD24 is expressed in schizont stage parasites

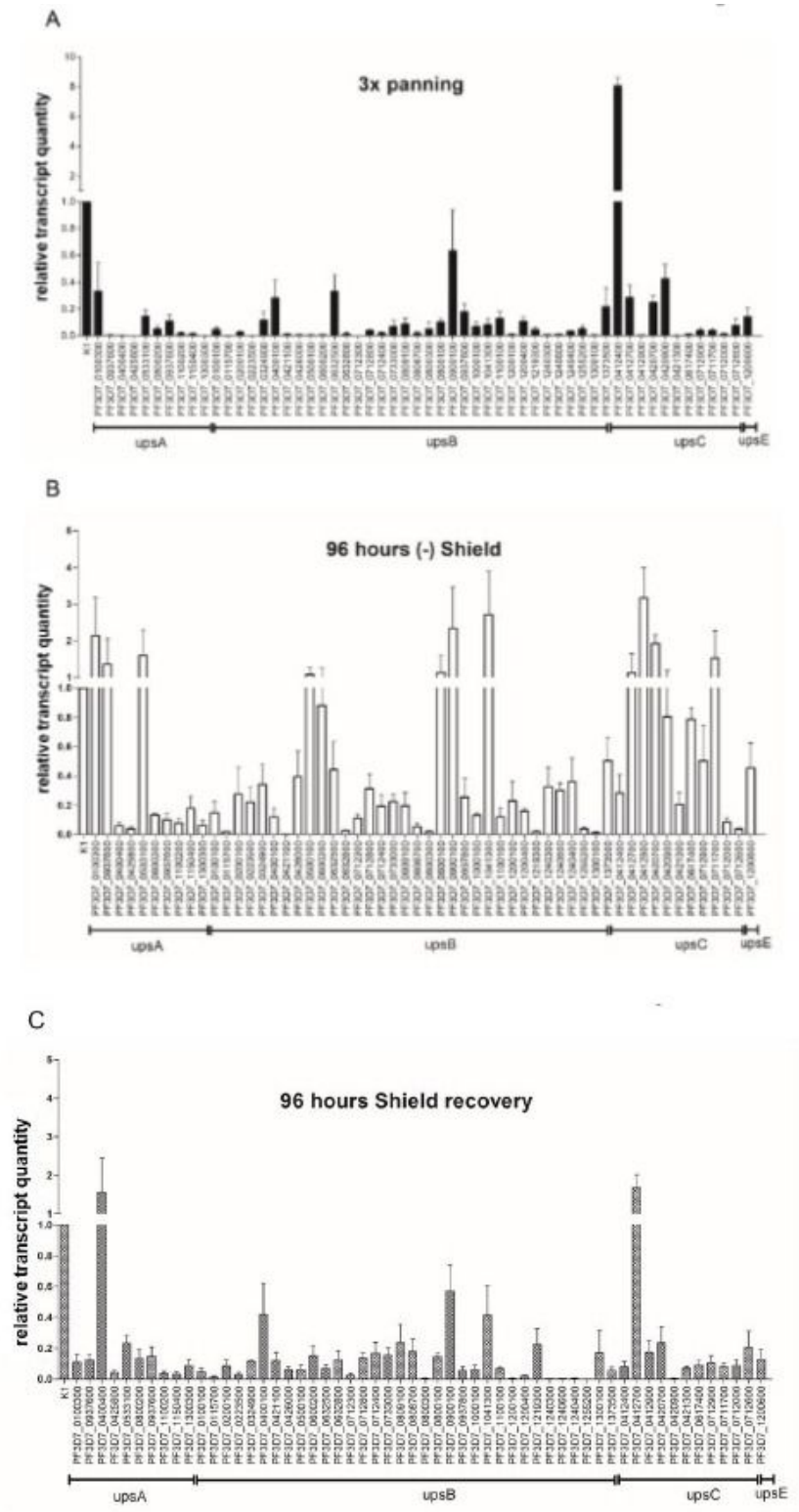

Figure 3

var gene transcription analysis during a transient knockdown in PfAP2-O-GFP-HA-DD24 parasites

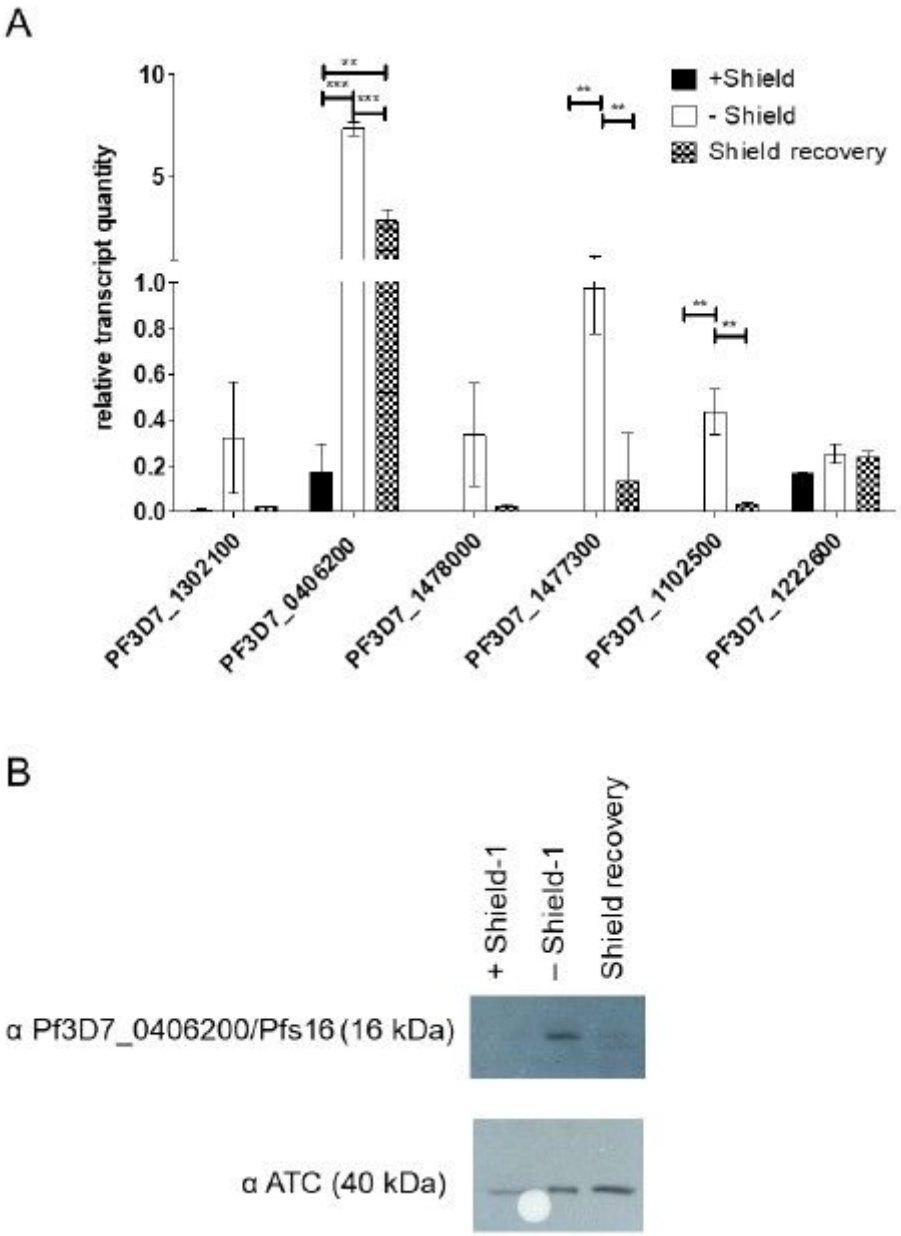

Figure 4

PfAP2-O-GFP-HA-DD24 is involved in the transcription/translation of gametocyte-related genes in P. falciparum

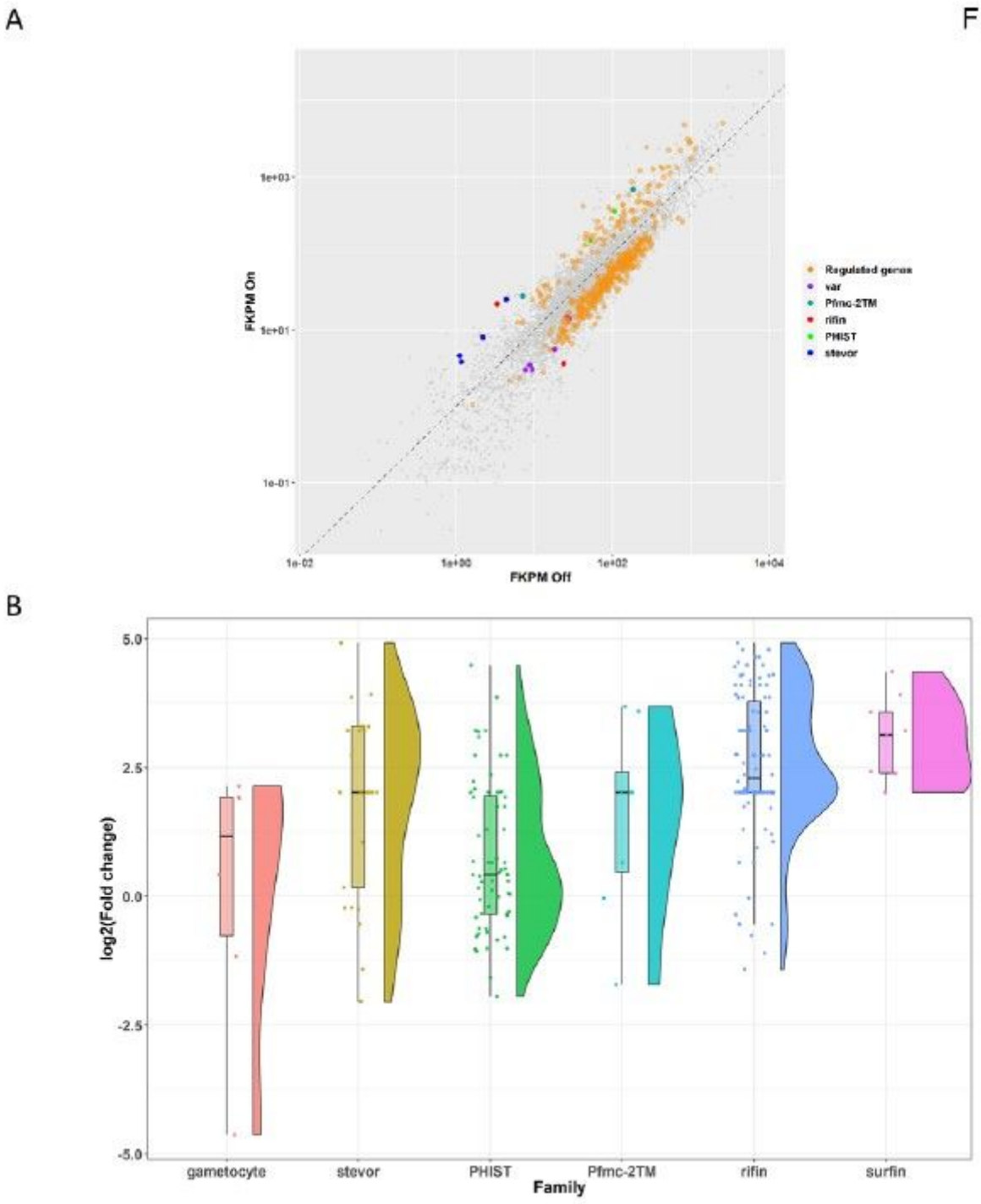

Figure 5

RNAseq analysis in PfAP2-O-GFP-HA-DD24 expressing or knocked down parasites in independent biological triplicates shows influence of PfAP2-O on varient gene transcripts

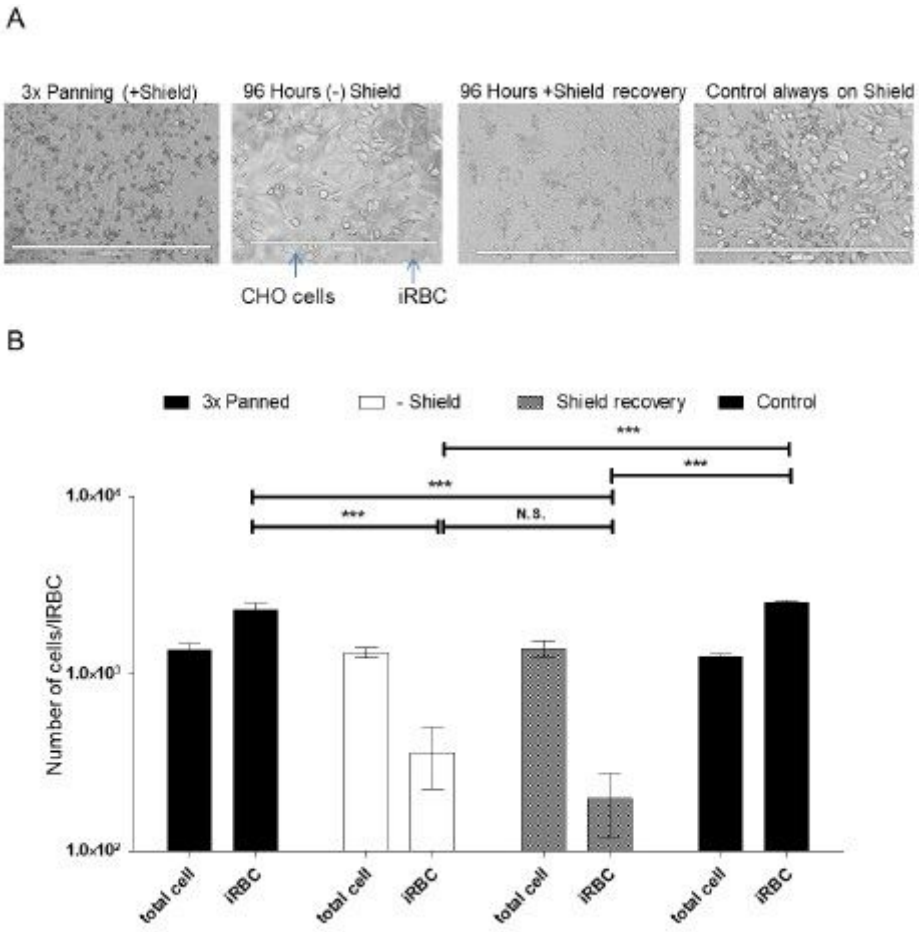

Figure 6

Adherent phenotype analysis shows decreased cytoadherence of IRBC during and after knockdown

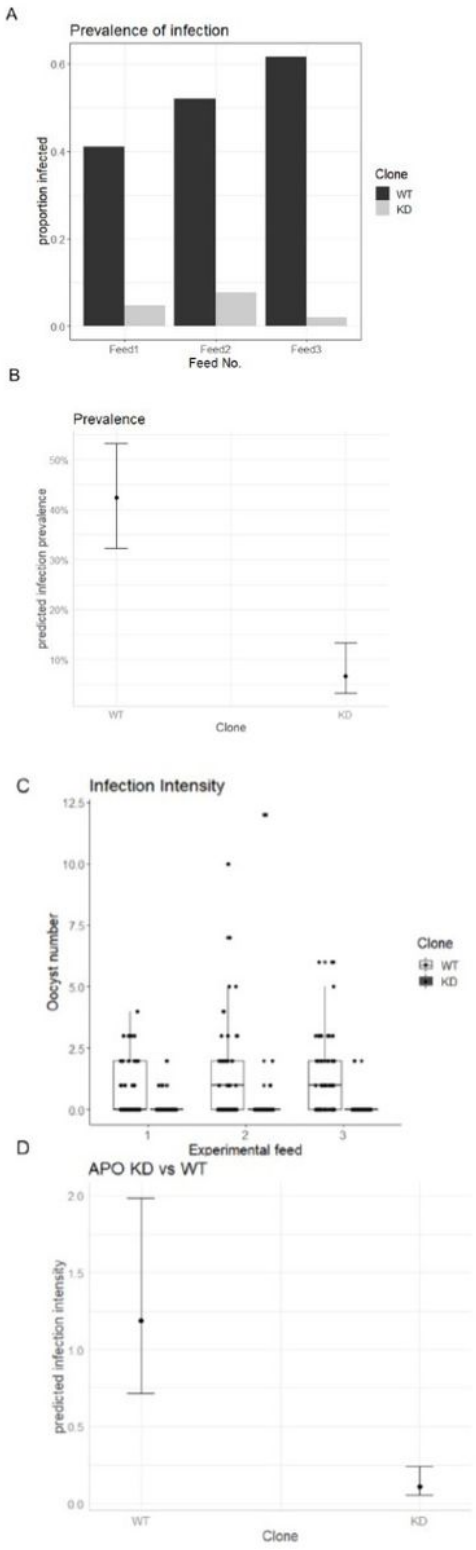

Figure 7

PfAP2-O is essential for P. falciparum transmission to Anopheles coluzzii

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementaryTable1.xlsx
- SupplementaryTable2.xlsx
- SupplementaryTable3RegulatedgeneswithTFmotifs.pdf
- SupplementaryTable4ProteomicAnalysis.pdf
- SupplementaryTable5RTPCRPrimers.xlsx
- SupplDataFile1.docx
- FigureS1.jpg
- FigureS2.jpg
- FigureS3.jpg
- FigureS4.jpg
- FigureS4a.jpg
- FigureS5.jpg