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ABSTRACT

The technical challenges of working with the sexual stages of the malaria parasite *Plasmodium* have hindered the characterization of sexual stage antigens in the quest for a successful malaria transmission-blocking vaccine. One such predicted and largely uncharacterized group of sexual stage candidate antigens is the CPW-WPC family of proteins. CPW-WPC proteins are named for a characteristic domain that contains two conserved motifs, CPxxW and WPC. Conserved across Apicomplexa, this family is also present earlier in the Alveolata in the free-living, non-parasitophorous, photosynthetic chromerids, *Chromera* and *Vitrella*. In *P. falciparum* and *P. berghei* blood stage parasites the transcripts of all nine *cpw-wpc* genes have been detected in gametocytes. RNA immunoprecipitation followed by reverse transcriptase-PCR reveals all *P. berghei cpw-wpc* transcripts to be bound by the translational repressors DOZI and CITH, and thus are likely under translational control prior to transmission from the rodent host to the mosquito vector in *P. berghei*. The GFP tagging of two endogenous *P. berghei* genes confirmed translational silencing in the gametocyte and translation in ookinetes. Establishing a luciferase transgene assay we show that the 3' untranslated region of PF3D7_1331400 controls protein expression of this reporter in *P. falciparum* gametocytes. Our analyses suggest that *cpw-wpc* genes are translationally silenced in gametocytes across *Plasmodium* spp. and activated during ookinete formation and thus may have a role in transmission to the mosquito.

Keywords: Translational repression; CPW-WPC; *Plasmodium*; malaria; gametocyte; ookinete

1. Introduction

Malaria is a debilitating disease caused by the apicomplexan parasite *Plasmodium* and transmitted by the female *Anopheles* mosquito. The disease is widespread in tropical regions, predominantly in South America, Southern Asia and Africa, with the highest prevalence in sub-Saharan Africa. While the total number of cases and mortality due to malaria has decreased significantly in the past few years, an excess of 200 million cases occurred in 2015, with an estimated 438,000 deaths attributed to the disease that year [1]. Over 90% of these occurred in Africa, primarily in children under five years old. This is predicted to be a conservative estimate, as malaria surveillance systems underperform due to inadequate resources in impoverished nations [2]. *Plasmodium* parasites alternate between cyclical, asexual replication in the mammalian host and sexual development in the anopheline vector. When an infected mosquito takes a blood meal, sporozoites are injected into the mammalian host; they establish infection in hepatocytes and replicate to release thousands of merozoites into the bloodstream causing disease. Merozoites typically undergo asexual replication within erythrocytes, but occasionally develop male and female gametocytes that are transmitted to the mosquito during a blood meal. Following fertilization, the resulting zygote transforms into a motile ookinete, which infects the vector and eventually gives rise to thousands of sporozoites that invade the mosquito's salivary glands, from where they can be transmitted to the next host.

In contrast to *in vitro* culture of intraerythrocytic asexual *P. falciparum* stages, the study of the sexual stages remains challenging due to its dependence on the use of mosquitoes and the resulting paucity and lack of purity of parasite material. Thus, many predicted sexual stage proteins remain uncharacterized and have unknown functions; specifically, of the 2430 proteins identified by mass-spectrometry in *P. falciparum* gametocytes (stages I-II, IV and V) [3, 4], 830 (34%) are annotated with 'unknown function' (www.plasmodb.org; June 5, 2014). Their molecular and cellular descriptions are vital to prioritize candidate surface proteins for transmission-blocking vaccine approaches that interfere with parasite development or inhibit infection of the mosquito vector by the ookinete [5]. Examples of such targets are the *P. falciparum* zygote and ookinete stage surface proteins Pfs25 and Pfs28 [6, 7].

In this manuscript we describe molecular studies of one family of putative transmission-blocking targets, the CPW-WPC protein family, which is characterized by a cysteine-rich protein domain structure and a phylogenetic distribution across the phylum Apicomplexa. Global expression data indicated sex-specific expression of the entire *cpw*-

wpc gene family in *P. falciparum* and *P. berghei* [8, 9] and four *P. yoelii cpw-wpc* genes were detected in gametocytes and young ookinetes [10]. Twenty-two *cpw-wpc* genes from three species are thus upregulated in gametocytes, suggesting a conserved role for this gene family during sexual development and transmission of the human and two rodent malaria species. A single member of the *P. yoelii* CPW-WPC protein family, PyCPW-WPC-1 (PY17X_1357700, ortholog of PBANKA_1352500 and PF3D7_1338800) was shown to be expressed on the surface of the ookinete stage parasite; its function was found to be redundant [10]. Like all CPW-WPC proteins PyCPW-WPC-1 has a predicted signal peptide, but lacks transmembrane domains or glycosylphosphatidyl inositol (GPI) anchors; association with the plasma membrane of the parasite [10] may require a complex with other surface proteins in a manner similar to the interaction of Pfs230 with Pfs48/45 [11] or the 6-member LCCL family in *Plasmodium* [12, 13]. Herein we establish that in *P. falciparum*, as well as the widely used rodent malaria model, *P. berghei*, the *cpw-wpc* family of genes is transcribed in gametocyte stages, but is likely translationally repressed until ookinete development in the mosquito midgut.

2. Materials and methods

2.1. Parasites

The *P. falciparum* NF54 isolate [14] was cultivated *in vitro* using RPMI 1640 medium supplemented with 10% heat-inactivated human serum and human erythrocytes at a 5% haematocrit as described [15]. Parasite development was synchronized by the isolation of schizonts using a Percoll-Sorbitol gradient and these parasites were placed back in culture. Thin smears of blood on glass slides were stained with Giemsa reagent for microscopy. Gametocytes were cultured using the method described by Fivelman et al. [16], with the addition of a treatment with 50 mM N-acetyl glucosamine to eliminate asexual stages.

The *P. berghei* genetically modified lines 683cl1 (WT DOZI::GFP; RMgm-133) expressing a C-terminally GFP-tagged version of DOZI (PBANKA_1217700), and line 909cl1 (WT CITH::GFP; RMgm-358) expressing a C-terminally GFP-tagged version of CITH (PBANKA_1301300) were used to obtain female gametocytes for the RNA immunoprecipitation (RIP) experiments as described [17]. Animal experiments performed at the University of Heidelberg Medical School (Heidelberg, Germany) were approved by the German Authorities (Regierungspräsidium Karlsruhe, Germany).

2.2. Reverse transcriptase-PCR (RT-PCR) of immunoprecipitated samples

Immunoprecipitation (IP) using *P. berghei* DOZI::GFP and CITH::GFP gametocyte extracts and subsequent RNA extraction, reverse transcription and PCR of IP fractions were carried out as described [17, 18]. Primers used in RT-PCR assays are shown in **Table 1**.

2.3. Plasmids

All plasmids used in the luciferase assay were derived from either pLN-ENR-GFP ([19]; www.mr4.org) or pHHT-TK ([20]; www.mr4.org). See **Table 2** for primer sequences and Figure 4 for construct design. The *Gaussia luciferase* gene (558 bp) was amplified from pCMV577-GLuc (New England BioLabs) using the primers 1 and 2 (**Table 2**), and inserted between the AvrII and AflII sites in pLN-ENR-GFP, flanked by either *calmodulin* (PF3D7_1434200) 5' and 3' flanking regions, or *calmodulin* 5' and *cpw-wpc* 3' flanking regions, which were amplified from *P. falciparum* strain NF54 genomic DNA. The 5' flanking region of *calmodulin* (982 bp) was obtained from the plasmid pLN-ENR-GFP [19]. The 3' flanking region of *calmodulin* (636 bp) was amplified from genomic DNA using the primers 3 and 4 [21]. The 3' flanking region of PF3D7_1331400 (967 bp) was amplified from genomic DNA using primers 5 and 6. The control plasmid carried the firefly *luciferase* gene amplified from the plasmid VLH1 [22] using the primers 7 and 8, and inserted between NotI and SpeI

sites on pHHT-TK. The firefly *luciferase* gene was flanked by *Pfs28* 5' (979 bp) and *Pfs28* 3' (400 bp) flanking regions, amplified from *P. falciparum* strain NF54 genomic DNA using the primers 9 and 10, and 11 and 12, respectively. All constructs were verified by sequencing.

2.4. Transfection of P. falciparum

P. falciparum NF54 parasites were transfected with the different luciferase reporter plasmids using the RBC-loading technique [23]. Stable transfection of parasites was obtained by pyrimethamine selection (40 ng/ml) of the human dihydrofolate reductase (*hDHFR)* expression cassette within the pHHT-TK plasmid, and blasticidin selection (2 µg/ml) for selection of the blasticidin-S-deaminase (BSD) cassette on the pLN-ENR-GFP plasmid.

2.5. Activation of PF3D7 gametocytes

Luciferase assays were performed with mature stage V gametocytes, harvested between days 14-16 of development. Late stage gametocyte cultures were pelleted by centrifugation and activated by resuspension of the parasite pellet in 1/10 volume of heat-inactivated A+ human serum (The Interstate Blood Bank, NYC, USA), and after 15 minutes incubation at room temperature exflagellation of male gametes was verified under the microscope. One hour following activation with serum the parasites were pelleted by centrifugation, resuspended in ookinete culture medium [RPMI 1640 pH 8.0, 20% FCS, 10% (v/v) penicillin-streptomycin-neomycin solution (GIBCO)], and were cultivated for 23 hours at 20°C. Luciferase activity of equivalent numbers of gametocytes was compared before activation and 24 hours post-activation.

2.6. Luciferase activity assay

Late stage gametocytes and ookinete cultures were washed once with PBS and frozen until use. The cells were thawed in Passive Lysis Buffer (Promega), and an equal volume of Renilla luciferase substrate (Renilla Luciferase Assay Kit, Promega) or firefly luciferase substrate (Firefly Luciferase Assay Kit, Promega) was added to the lysate, following which the activity was immediately measured using a TD-20/20 luminometer (Turner Designs). The luciferase activity of parasites from each transgenic line at each time point was measured in four independent experiments, and is depicted as percentage luciferase activity relative to activity of gametocytes before activation.

2.7. Generation and analysis of mutant P. berghei *expressing GFP-tagged CPW-WPC proteins*

The 3' end of the open reading frame (ORF) of PBANKA_1352500 was amplified with primers g3297 (TCTTTATTTATTTCTAGATATG) and g3294 (AAAGGATCCAATAATTGATCCGGTTATTGAATC); the 3' end of the PBANKA_1346300 ORF was amplified with primers g1248 (GATGGTATGTGCAATAGTG) and g3291 (AAAGGATCCTGACAATATATCAAAATCTGG). After digestion with HincII/BamHI or BstZ17I/BamHI each amplicon was ligated upstream and in frame of GFP in plasmid pLIS0010, producing pLIS0453 and pLIS0454. Downstream of the GFP cassette each plasmid contained the endogenous 3' UTR flanking region of PBANKA_1352500 [amplified with primers g3295 (AAATCTAGAATAACTTTTATTAGTATTTGTTTA) and g3296 (AAAGGTACCAATATATATTCATTATCTCC); cloned using XbaI/KpnI sites; 1107 bps] and PBANKA_1346300 [amplified with primers g3292 (AAATCTAGAATATGCGATATTTATGGATAG) and g3293 (AAAGGTACCTTTTATTTTTACAATATAATAAATG); cloned using XbaI/KpnI sites; 993 bps] respectively. Prior to transfection pLIS0453 was linearized with EcoRI, and pLIS0454 with BsmI, and transfected into *P. berghei* ANKA following established methodologies [24] resulting in the following mutant lines: T0453 (*pbanka_1352500::gfp*) and T0454 (*pbanka_1346300::gfp*). Integration of linearized plasmid DNA into the genomic locus was verified by PCR (**Figure S1**).

2.8. Live microscopy and Western blotting of blood stage P. berghei parasites and ookinetes

Gametocytes collected from mice infected with the transgenic *P. berghei* parasite lines T0453 or T0454 were incubated with 1 µg/mL of Hoechst-33342/PBS for live imaging of GFP fluorescence on a Zeiss 200M Axiovert widefield (63x) fluorescence microscope. Image processing was performed with ImageJ. Ookinete cultures were set up on day 5 post-infection; after cardiac puncture 0.6 mL blood was diluted in 10 ml ookinete culture medium and incubated at 19°C for 20 hours. 0.5 ml was then separated from the ookinete culture and incubated with Hoechst 33342/PBS and imaged as above. Red blood cells in the remainder of the ookinete culture were lysed in a 140 mM NH_4Cl solution on ice, ookinetes were collected by centrifugation and processed further for Western blotting with Roche monoclonal anti-GFP antibody (#11814460001).

2.9. RT-PCR of blood stage P. berghei parasites

Infected blood (0.2 ml) collected from mice infected with the transgenic *P. berghei* parasite lines T0453 or T0454 was treated on ice with saponin (14 mg/mL in PBS) to remove red blood cells; parasites were collected by centrifugation and processed for RNA isolation with QIAzol according to the manufacturer's recommendations. Following genomic DNA removal by DNase I, cDNA was prepared with Superscript II reverse transcriptase following

Invitrogen protocol and used in PCR in order to detect transcription of GFP-tagged alleles and known gametocyte transcripts [25]. Primer pairs were as follows: *pbanka_1342500::gfp* [3369 (GTGCCATGCCCAATTGG) and 178 (CCGTATGTTGCATCACCTTCACCC)]; *pbanka_1342500* [3369 (GTGCCATGCCCAATTGG) and 3294 (AATAATTGATCCGGTTATTGAATC)]; *pbanka_1346300::gfp* [3370 (AAACGGATATTGTTTAGCACC) and 178 (CCGTATGTTGCATCACCTTCACCC)]; *pbanka_1346300* [3370 (AAACGGATATTGTTTAGCACC) and 3291 (TGACAATATATCAAAATCTGG)]; *pbanka_0107700* [630 (AAAATTTTAGATGTGTTAG) and 584 (TTTTTTCACATTCAATTC)]; *pbanka_1114100* [639 (CAATTATTTTCATTGTC) and 640 (AAATAATCAATTTTGTTAAT)]; *pbanka_1334700* [637 (AATACTAATGTTTATGAC) and 638 (ATCTATAATGATATACTC)].

3. Results

3.1. CPW-WPC proteins are composed of arrays of a domain conserved throughout Apicomplexa

Members of the CPW-WPC family of proteins are characterized by the presence of one or more arrays of the CPW-WPC domain. Approximately 60 amino acids long, the domain is named for two conserved motifs; namely, CPxxW proximal to the amino-terminus and WPC proximal to the carboxy-terminus of the domain (**Figure 1A**). In some versions of the domain tryptophan is replaced with another aromatic residue, tyrosine or phenylalanine. Each domain typically contains 6 cysteines, likely paired in disulfide bridges, which is reminiscent of other cysteine-rich apicomplexan domains such as the COWP protein family of the oocyst wall in coccidians [26, 27]; the 6-Cys family that includes the *Plasmodium* gamete proteins Pfs230, Pfs47 and Pfs48/45 [28-31]; and the *Toxoplasma* SAG proteins [28, 30, 31]. The *cpw-wpc* gene family is widely conserved among apicomplexans, including *Plasmodium*, *Theileria*, *Babesia*, *Toxoplasma, Eimeria* and the gregarines (**Figure 1B**). The CPW-WPC proteins are absent in *Cryptosporidium*, suggesting that these genes were lost following the split of the cryptosporidian lineage. We identified amplifications of *cpw-wpc* genes in two chromerids, *Chromera* and *Vitrella* (**Figure 1B**), and thus the origin of the family predates the advent of obligate parasitism in the Apicomplexa. Amongst predicted extracellular proteins only the LCCL domain family (named for its first identification in metazoan proteins Limulus clotting factor C, Coch-5b2 and Lgl1) [32] is as widely distributed in Apicomplexa plus the chromerids *Chromera* and *Vitrella* [33-35]. Any proposed function of the CPW-WPC proteins must therefore take into account the natural histories of chromerid, apicomplexan and cryptosporidial protozoans in accordance with the presence or absence of CPW-WPC proteins. *Plasmodium* CPW-WPC proteins possess a variable number of CPW-WPC domains, ranging from 1 to 7; and they are randomly distributed across the protein with no preference for either the amino or carboxy terminus (**Figure 1C**). The *cpw-wpc* genes are conserved as orthologs across *Plasmodium*. The *cpw-wpc* gene family has 9 members in *P. falciparum* (**Figure 1C**), distributed across various chromosomes with some members encoded within a single exon (PF3D7_0320200 and PF3D7_1331400) while others are disrupted by 9 or more introns, with as many as 15 introns disrupting the ORF of PF3D7_1429300. To our knowledge, no other gene family in *Plasmodium* has such a wide disparity of gene structures within a single, amplified gene family. In *P. berghei* the gene structures are conserved with equal numbers of exons encoding each ortholog, as described (www.plasmodb.org). The absence of introns in *cpw-wpc* genes PF3D7_0320200 and PF3D7_1331400 are thus far unique to *P. falciparum* and their orthologs in *P. berghei*, *P. yoelii, P. vivax, P. chabaudi and P. knowlesi*. We did not identify examples of single exon

cpw-wpc genes in *Theileria*; *Babesia*; the coccidians; and the chromerids, *Chromera* and *Vitrella*. The individual *Plasmodium* members differ in length from 178 to 1256 amino acids. Sequence alignments and inferred phylogenetic trees of 54 members from six *Plasmodium* species (**Figure S2**), as well as BLAST analysis of best hits using *P. falciparum cpw-wpc* as queries, indicates that the respective *Plasmodium cpw-wpc* genes are conserved as orthologs across the *Plasmodium* genus. Therefore, the amplification of the gene family occurred prior to the split of *Plasmodium*, and the resulting genes remained orthologously conserved thereafter.

3.2. Translational repression of two CPW-WPC members in P. berghei *gametocytes is relieved during transmission*

Despite transcript evidence for all *P. falciparum* and *P. berghei* genes in late stage gametocytes, only a single protein (PF3D7_0624300) has been detected in the *P. falciparum* gametocyte proteome [4]; a single protein (PBANKA_1218300) was detected in *P. berghei* mixed male and female gametocytes represented by a single peptide [36] (**Table 3**). However, three *P. berghei* CPW-WPC members (PBANKA_1352500, PBANKA_1015400 and PBANKA_0943400) have been detected in the ookinete [37] indicating translational repression for these three (and perhaps all other) genes in a manner akin to the surface proteins P25 and P28; that is, both genes are transcribed in gametocytes but kept translationally quiescent until translated in the zygote and ookinete stage in *Plasmodium falciparum* and *P. berghei* [38-41]. Consistent with this theory, PyCPW-WPC-1 was shown to be transcribed in gametocytes but not translated until the zygote stage [10].

Transcripts of the genes *p25*, *p28* as well as *ap2-o*, a key transcription factor during ookinete maturation [42], are assembled into cytoplasmic mRNPs that can be visualized by FISH [18] and are co-immunoprecipitated with the repressors DOZI and CITH [17]. The DEAD-box RNA helicase DOZI (Development of Zygote Inhibited) [18] and the LSm protein CITH (CAR-I and Trailer Hitch homolog) [17] are core components of cytoplasmic messenger ribonucleoprotein particles (mRNPs) that function in the storage and stabilization of known repressed mRNAs in the female gametocyte, but are ready for rapid translation during ookinete formation after transmission of the parasite to the mosquito vector [17, 18, 38-40, 43]. Several *P. berghei cpw-wpc* transcripts were present in the pool of genes affected in cells depleted of DOZI and CITH (**Table 4**) [17, 18]. To test whether *cpw-wpc* mRNAs of *P. berghei* are bound in DOZI/CITH-defined mRNPs we used transgenic parasite lines that express GFP-tagged DOZI or CITH [17] in RNA immunoprecipitation (RIP) experiments (**Figure 2**). Anti-GFP antibodies were used to immunoprecipitate DOZI::GFP-

and CITH::GFP-containing complexes from enriched gametocyte preparations; mRNA was extracted and analyzed by RT-PCR with gene-specific primers and compared to known repressed and translated mRNAs. RT-PCR of the initial gametocyte extract (input lysate) identified all *cpw-wpc* and two control transcripts *p28* and *alba-3* in the gametocyte input fraction. *alba-3* transcripts that are translated in female gametocytes were not enriched in the immunoprecipitation (IP) fraction. Similar to *p28*, all *cpw-wpc* transcripts were bound by DOZI and CITH, corroborating their inclusion in DOZI/CITH-defined mRNPs that harbor translationally silent mRNA populations. The overall failure to detect CPW-WPC protein expression in *P. berghei* gametocytes [44] despite abundant transcript levels can therefore most likely be attributed to the sequestration of these mRNAs into mRNPs to be used during ookinete development.

We next developed two transgenic *P. berghei* lines expressing C-terminally tagged PBANKA_1352500 (transgenic line T0453) and PBANKA_1346300 (transgenic line T0454). Using established transfection methodologies [24] we introduced a GFP tag into each locus, thus providing wildtype transcriptional control and 5' flanking regions to the GFP-tagged gene; in order to maintain the wildtype 3' flanking context of each tagged gene, GFP in each plasmid was followed by approximately 1 kb of wildtype sequence (**Figures 3A/E and S2A**). Live fluorescence microscopy of both transgenic lines failed to show GFP protein expression in blood stage gametocytes (**Figures 3B/F**) although the gene was transcribed (**Figure 3C/G**); only ookinetes displayed GFP fluorescence (**Figure 3B/F**). The GFP-labeled CPW-WPC proteins appear to have intracellular localization in ookinetes, likely in crystalloid bodies, similar to that observed for the LCCL protein family in ookinete stages [45]. The LCCL and COWP protein families, along with the CPW-WPC family are conserved among pre-apicomplexan alveolates, and are likely to be markers for an archetypal pre-apicomplexan/ alveolate structure. It is thus plausible that the CPW-WPC proteins possess a function that is conserved in the alveolates *Chromera* and *Vitrella*. Similar to our observations, Kangwanrangsan et al. [10] observed gametocyte stage translational repression of one *P. yoelii* CPW-WPC proteins: PY17X_1357700, ortholog of PBANKA_1352500. However, using antibodies they observed surface expression of this and another CPW-WPC protein (PY03515, ortholog of PBANKA_1449300) in the zygote and ookinete stages, with no intracellular localization of these proteins. Using Western blot analysis we found PBANKA_1352500::GFP to be of the predicted size of 90 kDa, while PBANKA_1346300::GFP had a slightly smaller molecular weight than the predicted 82 kDa (**Figures 3D/H**).

3.3. A translational repression assay in P. falciparum *gametocytes identifies a conserved nature of cpw-wpc translational control*

After confirming the association of all *P. berghei cpw-wpc* transcripts with the translational repressors DOZI and CITH, and silencing of two GFP-tagged mutants in *P. berghei* gametocytes, we wished to evaluate a possible conserved nature of repression in *P. falciparum*. To test whether *P. falciparum cpw-wpc* genes could be under translational control prior to transmission to the mosquito vector we developed a luciferase assay, which is based on *P. berghei* experiments employing GFP as a transgene reporter, in which repression/translation in gametocytes of *gfp* relies on the presence/absence of specific 5' or 3' untranslated regions [46]. Instead of GFP we employed firefly and *Gaussia* luciferases [47-49] that use different substrates (D-luciferin for firefly luciferase, coelenterazine for *Gaussia* luciferase) and allow the detection of small changes in reporter gene expression. For control and experimental plasmids, genomic flanking regions provide endogenous promoter activity as well as the 3' UTR context.

We assessed luciferase reporter expression in gametocytes, and translation activation following gamete formation by comparing enzyme activities of an equivalent number of blood-stage *P. falciparum* gametocytes with those cultured for 24 hours at 20°C in ookinete medium. Under these conditions firefly luciferase under the control of the *Pfs28* 5' flanking region (containing the promoter) paired with the *Pfs28* 3' flanking region is robustly translationally upregulated following gametocyte activation as observed in the firefly luciferase assay (**Figure 4**). This is consistent with experiments performed in *P. berghei* and *P. falciparum* that detailed *p28* and *pfs28* repression mechanisms in gametocytes [41, 46]. Co-transfection of a plasmid carrying *Gaussia luciferase* flanked by the constitutively expressed *calmodulin* 5' flanking region (containing the promoter which is known to be active in asexual as well as sexual stage parasites) and the *calmodulin* 3' flanking region resulted in clear *Gaussia* luciferase activity in gametocytes parasites but no increase in ookinete culture extracts; on the contrary, we observed a 30% decrease following activation that we believe can be attributed to cell death in ookinete cultures.

Next we generated transgenic parasites carrying *Gaussia* luciferase under the control of the same *calmodulin* promoter/5' flanking region but this time paired with the 3' UTR of the *cpw-wpc* gene PF3D7_1331400. Parasite extracts showed a near three-fold increase in *Gaussia* luciferase activity following activation of gametocytes, establishing the presence of an element in the PF3D7_1331400 3' UTR that controls protein translation upon activation of gametocytes.

4. Discussion

In this manuscript we have detailed the evolutionary history of the CPW-WPC family of proteins. These proteins are ancient within Apicomplexa and are found in all genera except *Cryptosporidium*; and like the LCCL protein family their origin pre-dates the split of Apicomplexa to include the non-parasitic alveolates, *Chromera* and *Vitrella*. Because the *cpw-wpc* genes are predominantly transcribed in sexual stage *Plasmodium* parasites they are candidates for evaluation as transmission-blocking vaccine targets.

Using a luciferase assay we show that CPW-WPC protein expression in *P. falciparum* is regulated during transmission of the gametocyte. In *P. berghei* all nine members are bound by the translational repressors DOZI and CITH. Endogenous GFP tagging of two *P. berghei cpw-wpc* genes confirmed the presence of mRNA but absence of protein expression in the gametocyte, while protein translation was activated in the developing ookinete. Together with a recent study in *P. yoelii* [10] our data suggest an evolutionarily conserved role for this protein family during sexual development and parasite transmission, and this function appears to predate the Apicomplexa to include the alveolates *Chromera* and *Vitrella*.

It is worth noting key differences between *P. falciparum* and the *P. berghei* model for the study of gametocytes. Structurally, *P. falciparum* gametocytes adopt the namesake “falciform” sickle shape and develop over 12-15 days in culture [50, 51], whereas *P. berghei* gametocytes remain round throughout development, which is rapid and concomitant with asexual intraerythrocytic stages *in vivo* [52]. Mature *P. falciparum* gametocytes can circulate in the periphery of the human host for several weeks after their appearance [53, 54], while *P. berghei* gametocytes are viable only for 24-30 hours after maturation. Still, the short-lived *P. berghei* forms require a robust system of translational control to maintain certain transcripts in a stable, quiescent state to ensure readiness for rapid translation following activation of gametocytes in the mosquito midgut. Translational repression mediated by DOZI and CITH is essential for *P. berghei* ookinete development [17, 18, 25] and we believe that similar mechanisms play a role in the human parasite. In line with this hypothesis we have broadly defined a regulatory function for the 3’ flanking regions of two *P. falciparum* transcripts: *pfs28* and the *cpw-wpc* gene PF3D7_1331400 in gametocytes wherein translation is upregulated upon activation of gametocytes. Recognition of specific RNA motifs is key to translational repression in both *P. berghei* and *P. falciparum*. A U-rich region within the 3’ UTR of *P. berghei p28* [37] induces translational repression of a GFP reporter gene in female gametocytes [46], while in the case of *p25*, this role is played by its 5’ UTR [46]. Which

proteins bind these *P. berghei* motifs is unknown. These motifs have not been universally identified in putatively translationally repressed *P. berghei* genes and are absent in the *P. falciparum* orthologs of *p25* and *p28* [46]. Regulatory elements identified in the 3' UTR of *Pgs28*, the *P. gallinaceum* ortholog of *p28*, were shown to be essential for the expression of *Pgs28* [55, 56]. In contrast to the 5' UTR-mediated regulation of *p25*, the 3' UTR of *Pfs25* was shown to regulate luciferase reporter gene expression, and therefore hypothesized to regulate Pfs25 protein expression [57]. More recently PfPuf2 and Puf-binding elements located in the UTRs of *Pfs25* (5') and *Pfs28* (3') have been implicated in the translational repression of both transcripts [41]. Despite the lack of conservation of translational regulation of orthologous genes, the common, evolutionarily conserved denominator remains: the presence of a repressive element located in the 5' UTR and/or 3' UTR of a specific transcript [58-60].

A role for the *P. berghei* repressors DOZI and CITH in *P. falciparum* has not been demonstrated, but *cpw-wpc* transcripts could also be under the control of PfPuf2, a member of the RNA binding protein family Puf (*Drosophila melanogaster* Pumilio and *Caenorhabditis elegans* mRNA binding factor Fem-3) [61] which has a role in translational control of transmission-blocking candidates Pfs25 and Pfs28 [41]. In the absence of PfPuf2, transcript levels of translationally repressed genes are upregulated, and *pfs25* and *pfs28* are translated prematurely, not degraded [41], as is observed in the absence of DOZI or CITH in *P. berghei* [17, 18]. In line with this observation, transcripts of PF3D7_1338800, PF3D7_0320200 and PF3D7_1331400 *cpw-wpc* genes in *P. falciparum* are upregulated in *PfPuf2* knockout stage III gametocytes [41]. However, transcripts of the *cpw-wpc* genes PF3D7_1234700, PF3D7_1429300, PF3D7_0630000 and PF3D7_1331400 are downregulated in stage V *PfPuf2* knockout gametocytes, indicating that some other mode of translational repression is likely to regulate these genes. Ngwa et al. [62] report a decrease in the transcript levels of the *cpw-wpc* gene PF3D7_1103500 thirty minutes post-activation of gametocytes. However, translation of PF3D7_1103500 at this stage has not been demonstrated. Additionally, it has not been determined if the transcripts of all *cpw-wpc* genes are indeed translated *in vivo*, and several CPW-WPC proteins have not been detected in the available proteomic studies. It is also conceivable that CPW-WPC proteins have a short half-life *in vivo*, and are degraded rapidly after translation.

Using immunofluorescence Kangwanrangsan et al. [10] have demonstrated zygote and ookinete stage surface expression of two CPW-WPC proteins in *P. yoelii.* Disruption of one of these *cpw-wpc* genes in *P. yoelii* did not affect ookinete nor oocyst development. This result might be expected, if redundancy of expression and function in the CPW-WPC family

exists. Functional redundancy of the CPW-WPC family of proteins does not however make them less attractive as vaccine candidates. For example, P25 and P28 have partially redundant functions in the development of the sexual stages of the parasite in the mosquito midgut [63], but remain promising vaccine candidates. In order to characterize the CPW-WPC family as transmission-blocking vaccine candidates it will be important to include specific immune sera in transmission-blocking assays, as well as to pursue further description of the cellular localization and function of family members. The localization of two GFP-tagged CPW-WPC proteins described herein indicated intracellular localization in ookinetes, reminiscent of intracellular LCCL proteins in ookinetes described by Saeed et al. [45], although we did not characterize localization with respect to the crystalloid body as was observed for LCCL proteins in that study. The conservation of both LCCL and CPW-WPC proteins across the Apicomplexa plus the chromerids, *Chromera* and *Vitrella*, indicates that intracellular or cell surface expression of these proteins in ookinetes likely must be reconciled with a similar function in the chromerids.

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Figure legends

Fig. 1. Structure and phylogenetic distribution of CPW-WPC domain-containing proteins. (A) CPW-WPC domains are approximately 60 amino acids long and defined by a CPxxW motif at the N terminus and a WPC motif at the C terminus. Shown is an amino acid alignment of examples of CPW-WPC domains from *P. falciparum*, *Theileria parvum* (TP04_0183; contains three CPW-WPC domains); *Babesia bovis* (BBOV_III009100; contains two CPW-WPC domains); *Toxoplasma gondii* (57.m00035; contains five CPW-WPC domains); *Eimeria tenella* (ETH_00014705; contains three CPW-WPC domains); *Ascogregarina*; *Gregarina niphandrodes* (GNI_026410; contains 5 CPW-WPC domains); the chromerids, *Vitrella brassicaformis* (Vbra_13826; contains 5 CPW-WPC domains) and *Chromera velia* (Cvel_23115; contains 2 CPW-WPC domains); and select domains from two colpodellid genes. Domain numbers are indicated by d1, d2, etc., in order to distinguish multiple examples of CPW-WPC domains that are shown from a single protein. The location of each domain within the respective protein is indicated by amino acid position in parentheses. The *Ascogregarina* CPW-WPC sequence is data from a genome sequence survey and is a gene fragment. Conserved cysteine residues are highlighted in blue and conserved aromatic residues are highlighted in red. The position of the namesake amino acid residues, CPW and WPC are indicated in the line below the alignment. (B) The evolutionary conservation of *cpw-wpc* genes across Apicomplexa is shown on a phylogenetic tree adapted from Templeton et al. [64]. An asterisk (*) indicates that the genome sequence of the organism is incomplete. The *cpw-wpc* gene family appears to be present in all apicomplexans, with the exception of lineage-specific loss in *Cryptosporidium* (highlighted by the symbol 'X'). Protozoan parasites that do not have the *cpw-wpc* gene family are depicted in dark gray. The circled question mark indicates that the relationship of the chromerids to each other and within the alveolate clade has not yet been rigorously supported by phylogenetic analyses. (C) The nine members of the *cpw-wpc* gene family in *P. falciparum* are shown with gene identifiers, along with identification of predicted *P. berghei* orthologs. Gene structures are depicted with exons indicted by green rectangles. Protein domain architectures are also depicted, with predicted signal peptides represented by white rectangles, and a variable number of CPW-WPC domains represented by blue rectangles. Below the dotted line are gene and protein domain architectures for CPW-WPC protein examples from *Gregarina niphandrodes* (GNI_026410); and the chromerids, *Vitrella brassicaformis* (Vbra_13826) and *Chromera velia* (Cvel_23115). The lengths of all genes and proteins are to scale, and the PF3D7_1234700 gene is 3771 nt long, while the corresponding protein is 1256 aa long.

Fig. 2. CPW-WPC transcripts are associated with mRNPs defined by DOZI and CITH. DOZI- and CITH-containing mRNP granules were immunoprecipitated from DOZI::GFP and CITH::GFP transgenic parasites with anti-GFP antibodies. By RT-PCR all nine *cpw-wpc* transcripts were shown to co-elute with DOZI and CITH like the translationally repressed *p28*. *alba-3* serves as negative control as it is known to be translated in gametocytes and is not enriched in the IP-GFP fractions. input, total gametocyte mRNA; anti-myc, IP with anti-c-myc antibody; beads, no antibody used for IP.

Fig. 3. Translational repression of *P. berghei* CPW-WPC proteins during transmission. (A/E) Gene organization of PBANKA_1352500::GFP and PBANKA_1346300::GFP. (B/F) Live fluorescence microscopy of blood stage gametocytes and ookinetes show lack of protein expression in the former despite clear RT-PCR evidence (C/G) of transcription of both genes; control gametocyte transcripts are shown. Scale bar = 5μm. (D/H) Western blot of ookinete culture protein extracts. Asterisk (*) highlights the GFP-tagged proteins.

Fig. 4. Establishment of a luciferase translation repression assay for *P. falciparum* using *pfs28* regulatory sequences. *P. falciparum* parasites were co-transfected with control and reporter constructs. All parasites carried a control plasmid with firefly luciferase flanked by *pfs28* 5' and 3' flanking regions. Transgenic parasites carrying *Gaussia luciferase* flanked by *calmodulin (CAM)* 5' and 3' flanking regions showed an increase in firefly luciferase activity and a slight decrease in *Gaussia* luciferase activity 24 hours following activation of gametocytes. Transgenic parasites carrying *Gaussia luciferase* flanked by the *calmodulin* 5' flanking region and a *cpw-wpc* (PF3D7_1331400) 3' flanking region showed an increase in both firefly luciferase activity and *Gaussia* luciferase activity 24 hours following activation of gametocytes. Error bars represent standard deviation across four independent experiments for each transgenic line.

Fig. S1. *P. berghei cpw-wpc* GFP-tagging approach and genotyping. (A) Schematic representation of generic wildtype locus and GFP tagging plasmid, as well as the resulting mutant allele. Genotyping PCRs are indicated. (B) Genotyping of mutant PBANKA_1352500::GFP. Lane 1 (primers 3297 and 178) and lane 2 (primers 952 and 3296) amplify the regions outlined in A. Primers 3192 and 1142 amplify a control region. (C) Genotyping of mutant PBANKA_1346300::GFP. Lane 1 (primers 1248 and 178) and lane 2 (primers 952 and 3344) amplify the regions outlined in A. Primers 3192 and 1142 amplify a control region.

Fig. S2. Phylogenetic tree based on a multiple sequence alignment (ClustalOmega; http://www.ebi.ac.uk/Tools/msa/clustalo/) of 54 CPW-WPC proteins from 6 different *Plasmodium* species show that the respective *Plasmodium* proteins are conserved as orthologs across the genus. PCHAS *P. chabaudi*, PBANKA *P. berghei*, PY17X *P. yoelii*, PF3D7 *P. falciparum*, PKNH *P. knowlesi*, PVX *P. vivax*.

Table 1.

Gene-specific primer sequences for RT-PCR following RNA immunoprecipitation.

Gene ID	Primer type	Sequence
PBANKA_0943400	Sense	5'- ccccacttataaactgtcc
PBANKA_0943400	Antisense	5′- ctcatttggatggcaccctcc
PBANKA_1352500	Sense	5′- gggccgatatttgtgactc
PBANKA_1352500	Antisense	5′- catcgccatcttattccac
PBANKA_1218300	Sense	5′- gcttttgcctagctcctac
PBANKA_1218300	Antisense	5′- gggccgctaacaatggatag
PBANKA_1346300	Sense	5′- gatggtatgtgcaatagtg
PBANKA_1346300	Antisense	5′- gtttccctttacatggacc
PBANKA_1449300	Sense	5'- gtccaactggatggaatg
PBANKA_1449300	Antisense	5′- cccaattatcagggcatggg
PBANKA_1245200	Sense	5′- ccagaaaggagcattggtc
PBANKA_1245200	Antisense	5′- agctccagtcgcttagc
PBANKA_1015400	Sense	5'- gctatgcatggtgaattatcc
PBANKA_1015400	Antisense	5′- ccacactcatattccatgctc
PBANKA_1128800	Sense	5'- gctcgcatggaaatgtgaag
PBANKA_1128800	Antisense	5′- cattagcccatctagctcg
PBANKA_1123200	Sense	5'- ttatttgtgtcctgatgattg
PBANKA_1123200	Antisense	5′- gttatttggatttattgatcctc
PBANKA_0514900	Sense	5′- ttcgatatcatgaattttaaatacag
PBANKA_0514900	Antisense	5′- tccgcggccgcgcattactatcacgtaaataac
PBANKA_1204400	Sense	5′- aaacccggggaattccaagaaagagctgaaaac
PBANKA_1204400	Antisense	5′- aaagcggccgctattagcaacaaagtttg

Table 2.

Primer sequences for luciferase reporter constructs. FR: Flanking region.

Number	Sequence Amplified	Type	Sequence
1	*Gaussia* luciferase	Sense	5'- ggcctaggatgggagtcaaagttctgtttgcc
2	*Gaussia* luciferase	Antisense	5'- ggcttaagttagtcaccaccggccccttgat
3	Calmodulin 3' FR	Sense	5'- ggcttaaggatttttaaatatgcagaata
4	Calmodulin 3' FR	Antisense	5'- ggggatccgaacttaataaaaaagaggag
5	PF3D7_1331400 3' FR	Sense	5'- ggcttaagaacatatgaaaaattataaaataat
6	PF3D7_1331400 3'FR	Antisense	5'- ggggatccttgttgacgtgttgcatatataatatattcg
7	Firefly luciferase	Sense	5'- ggcctaggatggaagacgccaaaaacataaagaaagg
8	Firefly luciferase	Antisense	5'- ggcttaagttacaatttggactttccgccc
9	*Pfs28* 5' FR	Sense	5'- gggggcccgcggccgcaattcttctatttttttaattagctagctc
10	*Pfs28* 5' FR	Antisense	5'- ggcctaggtgtataaaaaactaaaaaatataaaataataagtg
11	*Pfs28* 3' FR	Sense	5'- ggcttaagaaaaaaaatatatatatatgtatatatatatatatatata tatatatatatatatagtcatatgatttgc
12	*Pfs28* 3' FR	Antisense	5'- ggggatccactagtaatataaattatctcattataatatttgcatatat

Table 3.

Summary of life cycle stage-specific proteomic data for CPW-WPC proteins.

Life cycle stage	Reference	CPW-WPC protein detected
P. berghei gametocyte	[36]	PBANKA_1218300
P. berghei ookinete	[37]	PBANKA_1352500 PBANKA_1015400 PBANKA_0943400
P. falciparum sporozoite	[65]	PF3D7_1234700 PF3D7_1331400
P. falciparum late stage gametocyte	[4]	PF3D7_0624300

Table 4.

Members of the *cpw-wpc* family are downregulated in DOZI or CITH deletion mutant *P. berghei* parasites.

P. berghei gene	WT vs. *dozi*-KO[a]	WT vs. *cith*-KO[a]
PBANKA_0943400	1.30	2.29
PBANKA_1015400	1.13	1.01
PBANKA_1128800	1.37	1.54
PBANKA_1218300	1.31	2.13
PBANKA_1245200	1.80	1.70
PBANKA_1346300	2.77	n.d.r.
PBANKA_1352500	1.76	2.02
PBANKA_1449300	1.37	1.29
PBANKA_1123200	n.d.r.	n.d.r.

[a]Microarray $\log_2$ ratios are depicted for *cpw-wpc* gene expression in wildtype (wt) compared to *dozi*-KO and *cith*-KO parasites. n.d.r. = not down regulated. Taken from Mair et al. [17, 18].

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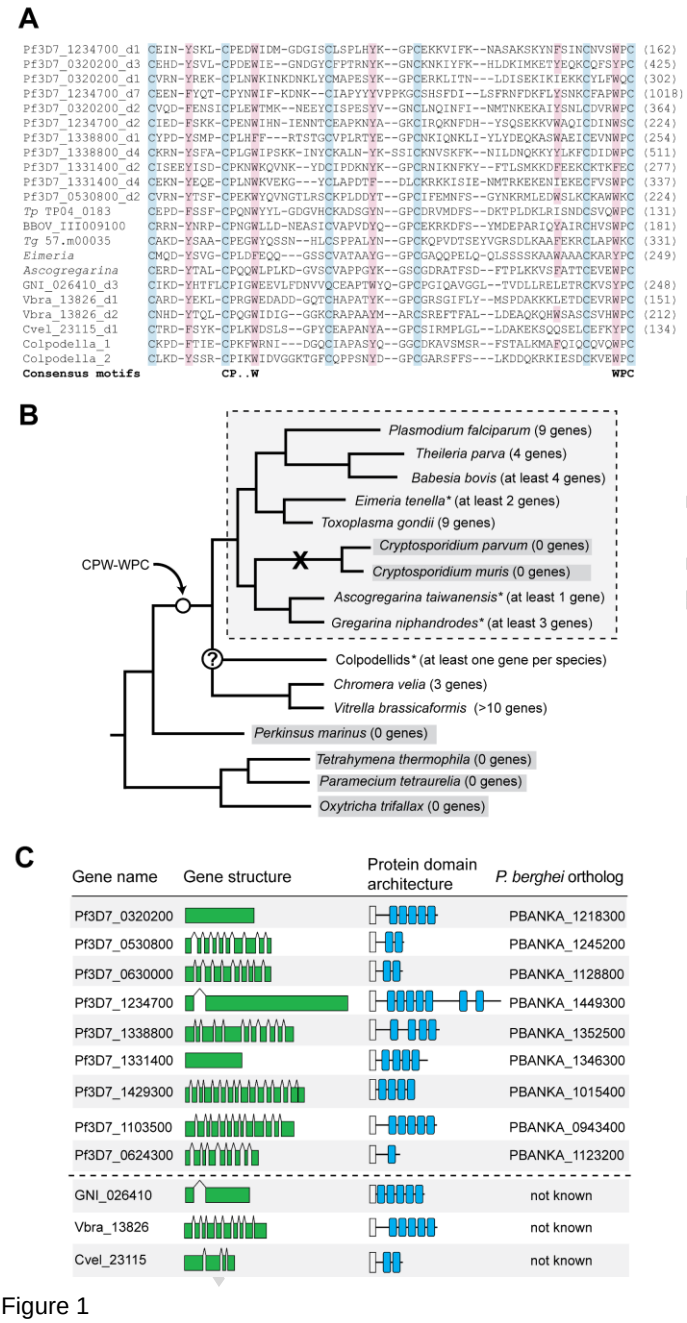


Figure 1

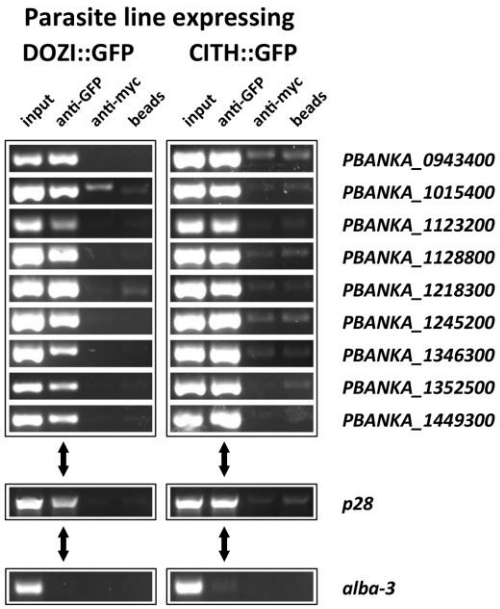


Figure 2

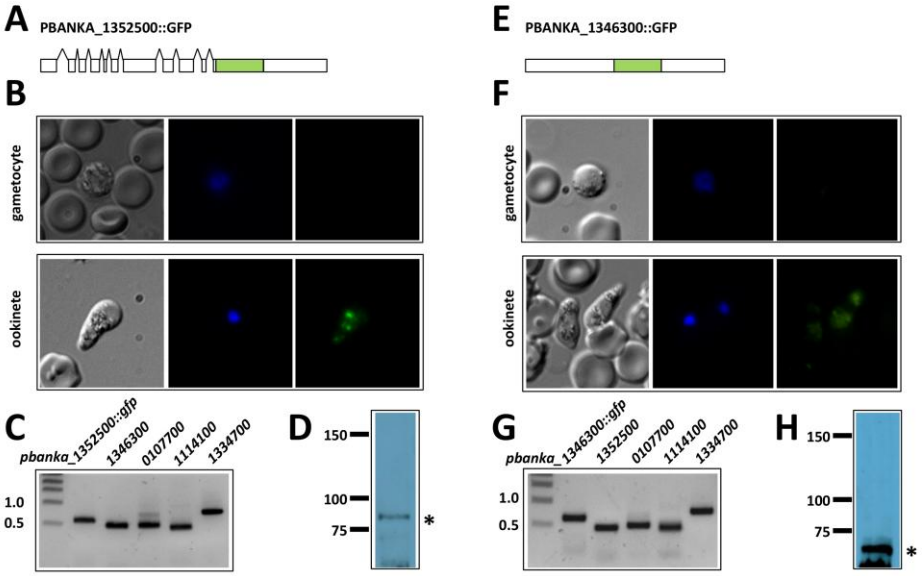


Figure 3

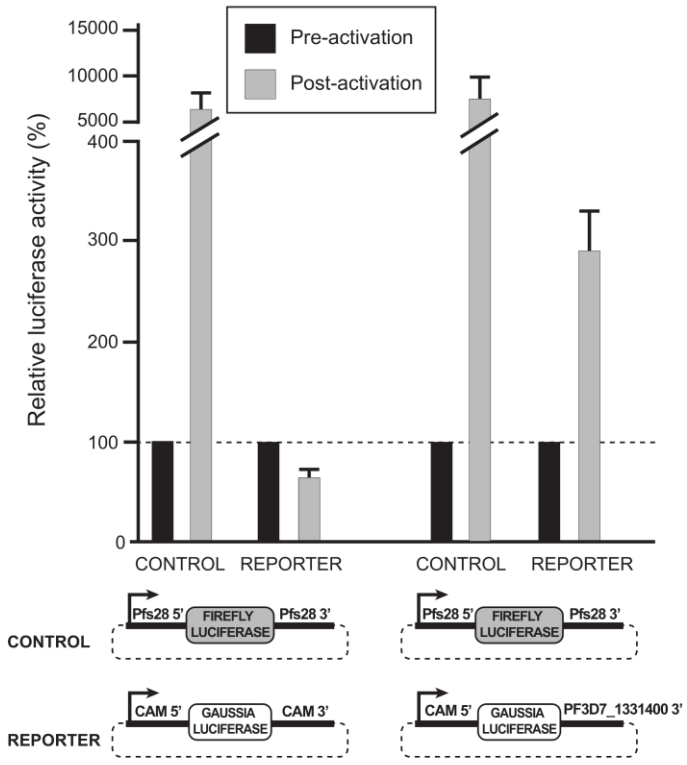
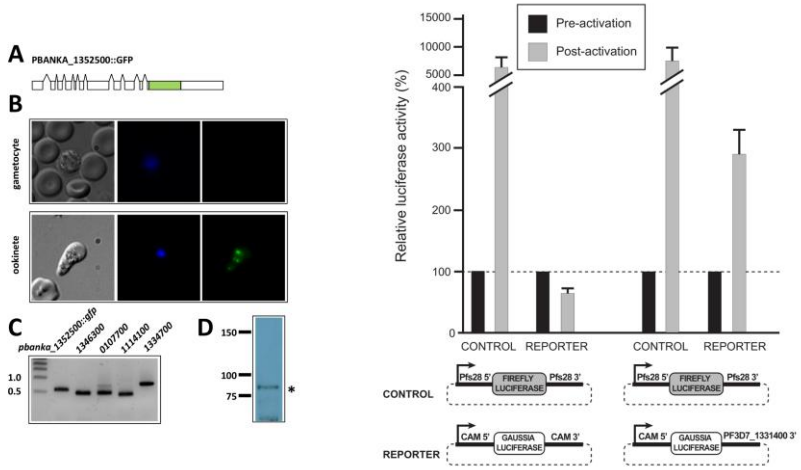


Figure 4

Graphical abstract



Highlights

- *P. berghei cpw-wpc* transcripts bound by translational repressors DOZI and CITH
- Translational silencing of two *P. berghei cpw-wpc* genes relieved in ookinetes
- Translational regulation by 3' untranslated region of a *P. falciparum cpw-wpc* gene