The WD40-Protein PfWLP1 Ensures Stability of the PfCCp-Based Adhesion Protein Complex in Plasmodium falciparum Gametocytes

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Members of the WD40-repeat protein family can be found in all eukaryotic proteomes where they usually serve as interaction platforms for the assembly of large protein complexes and are therefore essential for the integrity of these complexes. In the malaria parasite Plasmodium falciparum, the WD40-repeat protein PfWLP1 has been shown to interact with members of distinct adhesion protein complexes in the asexual blood stages and gametocyte stages. In this study, we demonstrate that the presence of PfWLP1 is crucial for both the stability of these gametocyte-specific adhesion complexes as well as for gametocyte maturation and gametogenesis. Using reverse genetics, we generated a PfWLP1-knockdown parasite line for functional characterization of the protein. Knockdown of PfWLP1 resulted in a slight reduction of gametocyte numbers and significantly the impaired ability of the gametocytes to exflagellate. PfWLP1-knockdown further led to reduced protein levels of the Limulus coagulation factor C-like (LCCL)-domain proteins PfCCp1 and PfCCp2, which are key components of the adhesion complexes. These findings suggest that the interaction of PfWLP1 with members of the PfCCp-based adhesion complex ensures complex stability and thereby contributes to gametocyte viability and exflagellation.

Keywords: malaria, Plasmodium falciparum, gametocyte, WD40-repeat protein, adhesion protein complex, CCp proteins

INTRODUCTION

The malaria parasite Plasmodium falciparum is transmitted from human to human through the bloodmeal of the mosquito vector. While symptoms in the human host are caused by asexual blood stage parasites, which multiply in the human red blood cells (RBCs), transmission to the mosquito is mediated by intraerythrocytic sexual precursor cells, the gametocytes. During their maturation in the human RBCs, the gametocytes pass through five morphologically distinguishable stages. When the mature gametocytes are taken up by the mosquito during its bloodmeal, they undergo sexual reproduction in the mosquito midgut and continue their life-cycle in the vector (reviewed in Kuehn and Pradel, 2010; Bennink et al., 2016).

The sexual phase of the malaria parasite goes along with the coordinated expression of stage-specific adhesion proteins, which assemble to complexes at the parasite plasma membrane. The
complexes include members of the *P. falciparum* LCCL domain-containing protein family (PfCCp) (Pradel et al., 2004; Pradel et al., 2006; Scholz et al., 2008; Simon et al., 2009; Simon et al., 2016; reviewed in Kuehn et al., 2010). The six PfCCp proteins, which are highly conserved among the apicomplexan clade (Pradel et al., 2004; Becker et al., 2010; Bastos et al., 2013; reviewed in Dessens et al., 2004), interact by adhesion domain-mediated binding and assemble to a complex with adhesive properties. The PfCCp-based protein complex is localized in the lumen of the parasitophorous vacuole (Pradel et al., 2004) and is anchored to the gametocyte plasma membrane via interactions with Pf\textit{s}230, which itself is binding to the GPI-anchored protein Pf\textit{s}48/45 (Kumar, 1987; Kumar and Wizel, 1992).

Upon gametocyte activation, coupling of the complex to the membrane is enhanced through the proteolytic processing of Pf\textit{s}230, additional protein-protein-interactions and the integration of the adhesion protein Pf\textit{s}25 into the complex, which further connects the complex with the plasma membrane via a GPI-anchor (Simon et al., 2016). These adhesion complexes are particularly present on the surface of the extracellular macrogametes that have formed during gametogenesis. It has been hypothesized that the PfCCp-based adhesion complex might play a role in adhesive processes during fertilization such as mediating contact between the female macrogamete and male microgamete (reviewed in Kuehn et al., 2010).

The six PfCCp proteins exhibit a co-dependent expression so that the lack of one PfCCp protein leads to the loss of other members of the family, while Pf\textit{s}230 expression is not affected (Simon et al., 2009). Further, the properly processed form of Pf\textit{s}230 is necessary for the correct assembly of the complex, since incorrect processing or lack of Pf\textit{s}230 leads to proteolysis of the PfCCp proteins and release from the complex in activated gametocytes (Brooks and Williamson, 2000; Simon et al., 2016).

Previously, the WD40-repeat protein-like protein Pf\textit{WLP1} has been identified as a component of the PfCCp-based protein complex in *P. falciparum* gametocytes (von Bohl et al., 2015). Pf\textit{WLP1} is composed of seven or multiple of seven WD40-repeats. The repeats are short repeating amino acid motifs with a length of approximately 44 to 60 amino acids which are terminated by a tryptophan-aspartate (WD) dipeptide (reviewed in e.g. Voorn and Ploegh, 1992; Neer et al., 1994; Xu and Min, 2011). WD40-domain containing proteins act as scaffolds for the formation of large protein complexes and are involved in numerous biological processes such as signal transduction, cell division or transcription regulation depending on their interaction partners (reviewed in Stirnimann et al., 2010; Jain and Pandey, 2018).

In the *P. falciparum* genome, 80 putative WD40-domain containing proteins have been identified in an in silico analysis, most of which remain uncharacterized until now (Chahar et al., 2015). Pf\textit{WLP1}, which has been demonstrated to be unique for the genus *Plasmodium*, has been shown to be expressed in the asexual schizont stage and during gametocyte development (von Bohl et al., 2015). In schizonts, the protein initially accumulates underneath the plasma membrane and subsequently relocalizes underneath the micronemes, when the merozoites have formed. In merozoites, Pf\textit{WLP1} then interacts with the transmembrane protein PfAMA1 (apical membrane antigen 1) – a part of the AMA1/RON (rhoptry neck protein)-complex, which is involved in tight junction formation during RBC invasion (Lamarque et al., 2011; reviewed in Boucher and Bosch, 2015). In the sexual stages, Pf\textit{WLP1} primarily localizes underneath the plasma membrane and associates with PfCCp1 and Pf\textit{s}230 in maturing and activated gametocytes (von Bohl et al., 2015).

In this study we aimed to analyse in more detail the link between Pf\textit{WLP1} and the PfCCp-based adhesion complex and to unveil the role of Pf\textit{WLP1} for gametocytes. To address the question, we generated a glms\textit{s}2-based Pf\textit{WLP1}-knockdown (KD) line and functionally characterized the protein using biochemical and cell-based assays.

**METHODS**

**Gene Identifiers**

The following PlasmoDB gene identifiers are assigned to the proteins investigated in this study: Pf\textit{39} [PF3D7_1108600], PfCCp1 [PF3D7_1475500], PfCCp2 [PF3D7_1455800], Pf\textit{s}230 [PF3D7_0209000], Pf\textit{WLP1} [PF3D7_1443400].

**Antibodies**

The following antisera were used in this study: mouse polyclonal antisera against PfCCp1rp1 (Scholz et al., 2008), Pf\textit{s}230 region C (Williamson et al., 1995), Pf\textit{39}rp1 (Simon et al., 2009) and Pf\textit{WLP1}rp1 (von Bohl et al., 2015); rabbit polyclonal antisera against PfCCp2 (Simon et al., 2009) and Pf\textit{s}230 (Biogenes); and polyclonal rabbit anti-HA antibody (Sigma-Aldrich).

**Parasite Lines**

In this study, the *Plasmodium falciparum* wild-type (WT) strain NF54 (ATCC) was used. The generation of the Pf\textit{WLP1}-KD line is described below.

**Parasite Culture**

The asexual blood stage parasites and gametocytes of the WT strain NF54 (WT NF54) and the Pf\textit{WLP1}-KD line were cultivated in human A+ RBCs in RPMI1640/HEPES medium (Gibco) supplemented with 10% v/v heat-inactivated human serum (A+). The medium was further complemented with 50 µg/ml hypoxanthine (Sigma-Aldrich) and 10 µg/ml gentamicin (Gibco). For cultivation of the Pf\textit{WLP1}-KD line, the selection drug WR99210 (Jacobus Pharmaceutical Company) was added in a final concentration of 2.5 nM. The cultures were kept at 37°C in an atmosphere of 5% O\textsubscript{2} and 5% CO\textsubscript{2} in N\textsubscript{2}. Human A+ RBCs and sera were purchased from the Institute of Transfusion Medicine, University Hospital Aachen, Germany. The donors remained anonymous and the RBC and serum samples were pooled. The work on human blood was approved by the Ethics Commission of RWTH Aachen University. Synchronization of
ring stage parasites was performed using sorbitol treatment as described (Lambros and Vanderberg, 1979). Gametocyte stages were enriched by Percoll gradient centrifugation (GE Healthcare Life Science) as previously described (Kariuki et al., 1998). In vitro induction of gametogenesis was performed by incubating mature gametocytes in 100 μM xanthurenic acid dissolved in 1% v/v 0.5 M NH₄OH/ddH₂O for 15 min at RT.

**Generation of PfWLP1-KD and Induction of Knockdown**

The PfWLP1-KD line was generated by single-crossover homologous recombination using the pARL-HA-glmS vector (Flammersfeld et al., 2020). A 1053-bp fragment homologous to the 3′ end of the pfwlp1 gene was amplified via PCR using forward primer 5′-TAGGCGGCGCGCAGATATTCAAATAATGGCATCTAC-3′ and reverse primer 5′-TAGGCGGCTAGGAAAAGCCAACAGCCCAAGAG-3′. Ligation of insert and vector backbone was mediated by NotI and AvrII restriction sites that were added to the PCR product via the primers. A synchronized WT NF54 ring stage culture was electroporated with 100 μg of the plasmid in transfection buffer (310 V, 950 μF, 12 ms; Bio-Rad gene pulser Xcell) as described (Wirth et al., 2014). WR99210 was added to the culture in a final concentration of 2.5 nM starting 6 h after transfection. Once resistant parasites appeared in the culture, they were checked for plasmid integration into the genome by diagnostic PCR. Therefore, genomic DNA of the transfected cultures was isolated using the NucleoSpin Blood Kit (Macherey-Nagel) and used as a template in the PCR. The following primers were used in the PCR (see Figure S1A for regions of primer binding): PfWLP1-KD-5′ integration forward primer 5′-CAATATATTGACAGCGGTTATGATGGG-3′ (primer 1), PfWLP1-KD-3′ integration reverse primer 5′-GTATATAATTTTCATGTTTTTAATATTGTACTCTC-3′ (primer 2), pARL-HA-glmS forward primer 5′-GCTTACACTTTATGCTTGCGCTCGGCT-3′ (primer 3) and pARL-HA-glmS reverse primer 5′-CTTATAGGCTCCGATAATCTGG-3′ (primer 4). To induce the glmS-ribozyme and thereby the knockdown of pfwlp1 gene expression, the culture was treated with 2.5 mM glucosamine hydrochloride (GlcN; D- (+)-glucosamine hydrochloride; Sigma-Aldrich) as described (Prommama et al., 2013).

**Growth Assays and Exflagellation Assay**

To investigate the asexual blood stage replication and gametocyte development, cultures of the WT NF54 strain or the PfWLP1-KD line were synchronized and set to an initial parasitemia of 0.25% or 2%, respectively. The parasites were cultivated either in the presence or absence of 2.5 mM GlcN. For the asexual growth assay, samples were taken and Giemsa-stained thin blood smears were prepared every 12 h over a time period of 96 h. For the gametocyte development assay, smears were prepared at five or six different time points over a time period of 13 to 15 d. The smears were evaluated microscopically at 1,000-fold magnification and parasitemia and gametocytemia were calculated by counting the percentage of parasites or gametocytes in 1,000 RBCs in triplicate, respectively. For the comparative exflagellation assay, gametocytes of the WT NF54 strain and the PfWLP1-KD line were cultivated in presence or absence of 2.5 mM GlcN for 12 to 14 days. Two days before the execution of the assay, GlcN was withdrawn from the cultures, since no exflagellation can be observed in GlcN-containing medium. The gametocytemia was adjusted between the samples to be compared and 100 μl of each culture was activated in vitro as described above. At 15 min post-activation, numbers of exflagellation centers were counted microscopically at 400-fold magnification in 30 optical fields.

**Western Blot Analysis**

While asexual blood stage parasites from WT NF54 and the PfWLP1-KD line were harvested from either mixed or synchronized cultures, gametocytes were enriched via Percoll gradient centrifugation (see above). Parasites were released from the enveloping RBC membrane by incubation in 0.05% w/v saponin/PBS for 10 min. Parasites were pelleted and resuspended in lysis buffer (150 mM NaCl, 0.1% v/v Triton X-100, 0.5% w/v sodium deoxycholate, 0.1% w/v SDS, 50 mM Tris-HCl pH 8.0) supplemented with protease inhibitor cocktail (complete EDTA-free, Roche). The lysates were supplemented with 5x SDS-PAGE loading buffer containing 25 mM dithiothreitol and heat-denatured at 95°C for 10 min. The protein lysates were separated via SDS-PAGE and subsequently transferred to a Hybond ECL nitrocellulose membrane (Amersham Biosciences). Blocking of non-specific binding sites was performed by incubation of the membrane in Tris-buffered saline containing 5% w/v skim milk, pH 7.5. For immunodetection, the membrane was incubated with polyclonal mouse anti-Pf39 antisera (dilution 1:2,500), mouse anti-PfCCp1 antisera (dilution 1:1,000), rabbit anti-PfCCp2 antisera (dilution 1:1,000) or rabbit anti-HA antibody (1:5,000) diluted in blocking solution at 4°C overnight. The membrane was washed and further incubated for 1 h at RT with a goat anti-mouse or anti-rabbit alkaline phosphatase-coupled secondary antibody (dilution 1:10,000, Sigma-Aldrich) and developed in a solution of nitroblue tetrazolium chloride (NBT) and 5-bromo-4-chloro-3-indoxylphosphate (BCIP, Sigma-Aldrich) for 5-15 min at RT. After being scanned, Western blots (WBs) were processed and band intensities were measured using the ImageJ 1.51f software.

**Indirect Immunofluorescence Assay**

Untreated and GlcN-treated gametocytes of the PfWLP1-KD line were air-dried as cell monolayers on glass slides and fixed in methanol at -80°C for 10 min. Membrane permeabilization and blocking of non-specific binding sites was performed by incubation in 0.01% w/v saponin/0.5% w/v BSA/PBS with 1% v/v neutral goat serum for 30 min at RT. For immunostaining, the preparations were incubated for 2 h at 37°C with polyclonal mouse anti-PfWLP1 antiserum (dilution 1:50), polyclonal mouse anti-PfCCp1 antiserum (dilution 1:50) or polyclonal rabbit anti-PfCCp2 antiserum (dilution 1:200) diluted in blocking solution. After washing, binding of the primary antibody was detected by incubation with Alexa Flour 488-conjugated goat anti-mouse or
goat anti-rabbit secondary antibody (Invitrogen) diluted 1:1,000 in PBS for 45 min at 37°C. Counterstaining of the gametocyte plasma membrane was performed by incubation with polyclonal rabbit anti-Pfs230 antiserum (dilution 1:500) or polyclonal mouse anti-PfH3230 antiserum (dilution 1:300) in blocking solution for 1 h at 37°C, followed by incubation for 45 min at 37°C with polyclonal Alexa Fluor 594-conjugated goat anti-rabbit or goat anti-mouse secondary antibody (Invitrogen) diluted 1:1,000 in PBS. Parasite nuclei were highlighted by treatment with Hoechst 33342 nuclear stain (Invitrogen) for 1 min at RT, Cells were mounted with anti-fading solution AF2 (CitiFlour™) and sealed with nail varnish. Microscopic evaluation was performed using a Leica DM 5500 B fluorescence microscope and fluorescence intensity was quantified using ImageJ 1.51f. Images were processed using Adobe Photoshop CS software.

**RESULTS**

**Generation of a PfWLP1-KD Parasite Line**

PfWLP1 was previously shown to be a 96-kDa cytosolic protein comprising five WD40 motifs that accumulates underneath the plasma membrane in gametocytes and here associates with members of the PfCCp-based adhesion protein complex (von Bohl et al., 2015). So far, the generation of a PfWLP1-knockout (KO) parasite line remains unsuccessful, although genetic manipulation of the pfwlp1 gene locus is possible (von Bohl et al., 2015). To characterize the role of PfWLP1 in the formation of the gametocyte specific adhesion protein complex in more detail, an inducible PfWLP1-KD line was generated, using the previously described pARL-HA-glmS vector (Flammersfeld et al., 2020). The pARL-HA-glmS-WLP1 vector was electroporated into ring stage parasites and WR99210 resistant parasites were obtained after six weeks. Integration of the vector into the pfwlp1 gene locus was confirmed by integration PCR (Figures S1A, B). Successful downregulation of pfwlp1 gene expression and in consequence reduced protein levels were confirmed by WB. For this, mixed asexual blood parasites of the PfWLP1-KD line were cultivated in the presence or absence of 2.5 mM GlcN for three days. Subsequent WB using a polyclonal anti-HA antibody confirmed the presence of the PfWLP1-HA fusion protein in lysates of the mutant line. Further, treatment with GlcN led to a reduction of PfWLP1-HA protein levels by approximately 45% (Figures S1C, D). Immunoblotting with polyclonal mouse anti-Pf39 antisera was used as a loading control and for normalization of band intensities. To confirm the downregulation of PfWLP1 in the gametocyte stages, gametocytes of the PfWLP1KD-line were cultivated in the presence or absence of 2.5 mM GlcN for 14 d, followed by WB analysis and quantification of the PfWLP1-HA-specific band intensities was performed as described above. Similar to the asexual blood stages, GlcN-treatment in gametocytes led to a significant reduction of PfWLP1-HA protein levels by approximately 40% (Figures 1A, B). Reduction of PfWLP1-HA protein levels in gametocytes upon GlcN-treatment was further confirmed via immunofluorescence assay (IFA), where even a reduction of the protein levels by approximately 75% was detected (Figures 1C, D). To investigate, if the reduction of the PfWLP1-HA protein levels affect the morphology of the parasites, thin blood smears were prepared from WT NF54 and PfWLP1-KD cultures that were cultivated either in the presence or absence of 2.5 mM GlcN. The smears were Giemsa-stained and evaluated by light microscopy. The treatment with GlcN did not have a visible effect on the morphology of either WT NF54 or PfWLP1-KD blood stage parasites. Both, asexual blood stages and gametocytes at different stages of maturity exhibited normal morphologies after GlcN-treatment (Figure 1E).

**PfWLP1 Is Crucial for Exflagellation**

Comparative phenotype analyses between WT NF54 and the PfWLP1-KD line cultivated either in the presence or absence of 2.5 mM GlcN showed that downregulation of PfWLP1-HA does not have a significant effect on the asexual blood stage replication (Figures 2A, Figure S1E). However, GlcN-treated PfWLP1-KD parasites showed a significant reduction in gametocyte numbers formed after 13 d of cultivation compared to untreated parasites (Figure 2B), while GlcN-treatment did not affect gametocyte formation in WT NF54 parasites (Figure S1F), indicating that PfWLP1 impacts gametocyte maturation. Hence, the ability of the parasites to form motile male microgametes was analyzed in exflagellation assays. Therefore, WT NF54 and PfWLP1-KD gametocytes were grown in GlcN-containing medium until maturity. Two days prior to gametocyte activation, GlcN was withdrawn from the cultures. After in vitro activation of the gametocytes, the numbers of exflagellation centers were evaluated by light microscopy. GlcN-treatment resulted in a significant inhibition of exflagellation in PfWLP1-KD gametocytes when compared to GlcN-treated WT NF54 gametocytes (Figure 2C), pointing to a crucial role of PfWLP1 in exflagellation. Untreated PfWLP1-KD and WT NF54 gametocytes on the other hand exhibited comparable numbers of exflagellation centers in this experiment (Figure 2C).

**Knockdown of PfWLP1 Leads to Reduced Expression of Members of the PfCCp-Complex**

Previous studies unveiled a co-localization and interaction of PfWLP1 with members of the PfCCp-complex (von Bohl et al., 2015). With the help of the PfWLP1-KD line we now aimed at investigating a potential effect of PfWLP1 on the integrity and stability of the PfCCp-based adhesion protein complex. Therefore, gametocytes of the PfWLP1-KD line were treated with GlcN to induce KD of PfWLP1 and protein levels of selected members of the PfCCp-complex (i.e. PfCCp1 and PfCCp2) were evaluated. In IFA, immunolabelling for both, PfCCp1 and PfCCp2 was significantly reduced in GlcN-treated PfWLP1-KD gametocytes compared to untreated gametocytes (Figures 3A, B). However, the expression pattern analysis showed a typical punctuated localization of the remaining PfCCp1 and PfCCp2 on the gametocyte plasma membrane, as it was previously described (Pradel et al., 2004; Pradel et al., 2006). Noteworthy, the
immunolabeling for \textit{Pf}s230, which was used for counterstaining, did not alter between the treated and untreated gametocytes, although it is also part of the \textit{Pf}CCp-protein complex (Scholz et al., 2008; Simon et al., 2009; reviewed in Kuehn et al., 2010). In WT NF54 gametocytes, GlcN-treatment did not have an effect on the intensity of \textit{Pf}CCp1- and \textit{Pf}CCp2-fluorescence signals (Figures S2A, B). \textit{Pf}CCp1 and \textit{Pf}CCp2 protein levels were further compared in protein lysates of GlcN-treated and untreated \textit{Pf}WLP1-KD gametocytes via WB followed by quantification of band intensities. Immunoblotting and quantification revealed that the \textit{Pf}CCp1- and \textit{Pf}CCp2-specific protein bands were significantly less intense in the GlcN-treated samples compared to the untreated samples (Figures 3C, D). Immunoblotting with antisera against \textit{Pf}39 served as a loading control and was used for normalization of the band intensities. The combined data demonstrate that the lack of \textit{Pf}WLP1 results in a reduction of \textit{Pf}CCp proteins on the gametocyte plasma membrane.

**DISCUSSION**

Apicomplexan parasites exhibit various types of adhesive protein complexes on the cell membranes, which exert distinct functions and which depend on the life-cycle stage of the parasite. These adhesive multi-protein complexes are utilized by the parasites for different types of intercellular contact, ranging from gliding and attachment to target cells to host cell invasion and cytoadherence of infected RBCs. In the malaria parasite \textit{P. falciparum}, membrane-coupled protein complexes composed of transmembrane proteins as well as GPI-anchored and peripheral adhesion proteins are known for the different life-
cycle stages of the parasite. One example is the merozoite surface protein (MSP)-based protein complex, which is essential for the successful attachment of the merozoites to RBCs prior to invasion (reviewed in Cowman and Crabb, 2006). Merozoites further express the PfAMA1/RON complex, which defines the merozoite-RBC moving junction (e.g. Collins et al., 2009; Richard et al., 2010; Srinivasan et al., 2011). PfAMA1 is stored in the micronemes and translocated to the merozoite surface before invasion, while at the same time, the RON-complex is inserted into the host cell membrane (Cao et al., 2009; Riglar et al., 2011; reviewed in Shen and Sibley, 2012).

An adhesion complex similar in its structure to the PfMSP-based complex is found in gametocytes and gametes. The multi-protein complex is based on the six members of the LCCL-domain/PfCCp protein family and further includes the 6-cys proteins Pf6230 and Pf648/45, the latter of which is linked to the membrane via a GPI anchor. In addition, the EGF-domain protein Pf625 links the complex to the macrogamete surface (e.g. Simon et al., 2009; Simon et al., 2016; reviewed in Kuehn et al., 2010). The adhesive PfCCp-based protein complex on the surface of the gametes is crucial for sexual reproduction. Interestingly, (KO) of the PfCCp-proteins does not abolish exflagellation in the respective KO-lines, but impairs the transition of midgut sporozoites to the salivary glands (Pradel et al., 2004; Scholz et al., 2008). Pf6230-KO parasites are also able to egress from the host RBC and exflagellate, but are unable to interact with surrounding cells and form exflagellation centers (Eksi et al, 2006).

In addition to the adhesion proteins on the gametocyte plasma membrane, the cytosolic WD40-domain protein PfWLP1 has previously been described as an interaction partner of the PfCCp-protein complex (von Bohl et al., 2015). PfWLP1 is a member of the WD40-domain containing protein family which is one of the most abundant protein classes in eukaryotes and also includes prokaryotic proteins. Out of the 80 putative WD40-domain-containing proteins that have been identified in the *P. falciparum* genome (Chahar et al., 2015), only a few have been partially characterized so far, including for example the nuclear pore complex component PfSec13, which is also involved in the biogenesis of COPII-coated vesicles (Dahan-Pasternak et al., 2013), PfRACK, an ortholog of the receptor for activated C kinase (Madeira et al., 2003) and the histone chaperone PfRbAp46/48 (Kausik et al., 2020). PfWLP1 was shown to be expressed in the maturing schizont as well as the gametocyte stages, where it accumulates underneath the plasma membrane and interacts with the stage-specific PfAMA1/RON- and PfCCp-complexes, respectively (von Bohl et al., 2015).

In this study we have successfully generated a PfWLP1-KD line, in which the PfWLP1 protein levels can be reduced by approximately 40-45% via GlcN-mediated transcript degradation, as detected in WB. The KD did not show any...
growth defect during the asexual replication cycle, pointing to a non-essential role of *Pf*WLP1 in schizogony, contrary to assumptions in previous studies, in which a gene-KO remained unsuccessful (von Bohl et al., 2015) and a piggyBac transposon mutagenesis study that confirmed essentiality of *Pf*WLP1 (Zhang et al., 2018). These contradictory data might be explained by the low KD rate, in asexual blood stages after 3 d of GlcN-treatment. Longer GlcN-treatment could lead to higher KD rates which might result in growth defects.

In the *Pf*WLP1-KD, gametocyte maturation was affected and exflagellation was severely impaired when compared to WT NF54 parasites. Interestingly, the exflagellation ability is not affected in KO s of *Pf*CCp1 – an interaction partner of *Pf*WLP1 – or other *Pf*CCp proteins. Noteworthy, antibodies against *Pf*CCp1-4 and *Pf*FNPA are able to inhibit exflagellation, however this effect is only measurable in active serum and not in heat-inactivated serum, pointing to a participation of active complement in the process (Scholz et al., 2008).

We further showed that the KD of *Pf*WLP1 leads to reduced abundance of members of the *Pf*CCc-complex, i.e. its interaction partners *Pf*CCp1 and *Pf*CCp2. Noteworthy, in the GlcN-treated *Pf*WLP1-KD the remaining *Pf*CCp1 and *Pf*CCp2 proteins showed a similar subcellular localization as in the untreated culture, indicating that the *Pf*CCc-complex still assembles and localizes correctly in the *Pf*WLP1-KD line. However, low levels of *Pf*CCc proteins are still present on the gametocyte surface, which might also be explained by the fact, that only roughly 45% of *Pf*WLP1 was downregulated. Interestingly, the expression of *Pfs*230, which is also a member of the *Pf*CCc-based protein complex, was not affected in the *Pf*WLP1-KD. Similar results were obtained when the co-dependent expression of the *Pf*CCc-proteins was analysed. While KO of one *Pf*CCc-protein led to the complete or partial loss of other members of the protein family, the expression and localization of *Pfs*230 was not affected, indicating that *Pfs*230 expression is not dependent on the presence and assembly of all complex members (Pradel et al., 2006; Simon et al., 2009; reviewed in Kuehn et al., 2010).

Based on these data we suggest that the cytosolic *Pf*WLP1 serves as an interaction platform for the *Pf*CCc-based adhesion protein complex in gametocytes and gametes and is important for its assembly and/or integrity. We hypothesize that the intracellular *Pf*WLP1 binds to the *Pf*CCc-complex via an unknown membrane-spanning linker protein, which binds to one or more *Pf*CCc-proteins in the lumen of the parasitophorous vacuole. The complex is bound to *Pfs*230 via interactions of *Pfs*230 and *Pf*CCc4 (see Figure 4). As *Pf*WLP1 was described to localize in close proximity with sub-pellicular proteins (von Bohl et al., 2015), it might connect the membrane-linked protein complex with the inner membrane complex of the parasite.

Since several members of the *Pf*CCc-complex have been proposed and investigated as putative antigens for the
development of transmission-blocking vaccines, further analysis of the constituents and interactions of this complex is of particular importance.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

LR: methodology and data analysis. AF: methodology and data analysis. SB: data analysis and visualization. GP: conception and project administration. SB and GP: writing original draft. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2022.942364/full#supplementary-material

REFERENCES

Bastos, R. G., Suarez, C. E., Laughery, J. M., Johnson, W. C., Ueti, M. W., and Knowles, D. P. (2013). Differential Expression of Three Members of the Multidomain Adhesion CCP Family in Babesia Bigemina, Babesia Bovis and Theileria Equi. PloS One 8, e67765. doi: 10.1371/journal.pone.0067765

Becker, C. A. M., Malandrin, L., Depoix, D., Larcher, T., David, P. H., Chauvin, A., et al. (2010). Identification of Three CCP Genes in Babesia Divergens: Novel Markers for Sexual Stages Parasites. Mol. Biochem. Parasitol. 174, 36–43. doi: 10.1016/j.molbiopara.2010.06.011

Bennink, S., Kiesow, M. J., and Pradel, G. (2016). The Development of Malaria Parasites in the Mosquito Midgut. Cell. Microbiol. 18, 905–918. doi: 10.1111/cmi.12604

Boucher, L. E., and Bosch, J. (2015). The Apicomplexan Glideosome and Adhesins – Structures and Function. J. Struct. Biol. 190, 93–114. doi: 10.1016/j.jsb.2015.02.008

Brooks, S. R., and Williamson, K. C. (2000). Proteolysis of Plasmodium falciparum Surface Antigen, Pfs230, During Gametogenesis. Mol. Biochem. Parasitol. 106, 77–82. doi: 10.1016/S0166-6851(99)00201-7

Cao, J., Kaneko, O., Thongkhuatkul, A., Tachibana, M., Otsuki, H., Gao, Q., et al. (2009). Rhoptyr Neck Protein RON2 Forms a Complex With Microneme Protein AMA1 in Plasmodium falciparum Merozoites. Parasitol. Int. 58, 29–35. doi: 10.1016/j.parint.2008.09.005

Chahar, P., Kaushik, M., Gill, S. S., Gakhar, S. K., Gopalan, N., Datt, M., et al. (2015). Genome-Wide Collation of the Plasmodium falciparum WDR Protein Superfamily Reveals Malarial Parasite-Specific Features. PloS One 10, e0128507. doi: 10.1371/journal.pone.0128507

Collins, C. R., Withers-Martinez, C., Hackett, F., and Blackman, M. J. (2009). An Inhibitory Antibody Blocks Interactions Between Components of the Malarial Invasion Machinery. PloS Pathog. 5, e1000273. doi: 10.1371/journal.ppat.1000273

Cowman, A. F., and Crabb, B. S. (2006). Invasion of Red Blood Cells by Malaria Parasites. Cell 124, 755–766. doi: 10.1016/j.cell.2006.02.006

Dahan-Pasternak, N., Nasereddin, A., Kolevzon, N., Pe’er, M., Wong, W., Shinder, V., et al. (2013). Pfsct13 Is an Unusual Chromatin-Associated Nucleoporin of Plasmodium falciparum That Is Essential for Parasite Proliferation in Human Erythrocytes. J. Cell Sci. 126, 3055–3069. doi: 10.1242/jcs.122119

Desens, J. T., Sinden, R. E., and Claudians, C. (2004). LCCL Proteins of Apicomplexan Parasites. Trends Parasitol. 20, 102–108. doi: 10.1016/j.pt.2004.01.002

Elki, S., Czesny, B., van Gemert, G.-J., Sauerwein, R. W., Eling, W., and Williamson, K. C. (2006). Malaria Transmission-Blocking Antigen, Pfs230, Mediates Human

FIGURE 4 | Model of the composition of the PICCCp-based protein complex in WT NF54 gametocytes and proposed changes in PWLP1-KD gametocytes. Intracellular PWLP1 probably binds to the PICCCp-complex via an unknown membrane-spanning linker protein (L). In the absence of PWLP1, the PICCCp-complex is destabilized, while Pfs230 remains unaffected (destabilized complex shown in grey). PPM, parasite plasma membrane; PVM, parasitophorous vacuole membrane.
Red Blood Cell Binding to Exflagellating Male Parasites and Oocyst Production. *Mol. Microbiol.* 61, 991–998. doi: 10.1111/j.1365-2958.2006.05284.x

Flammersfeld, A., Panayot, A., Yamaryo-Botté, Y., Aurass, P., Przyborski, J. M., Flieger, A., et al. (2020). A Patatin-Like Phospholipase Functions During Gametocyte Induction in the Malaria Parasite Plasmodium falciparum. *Cell. Microbiol.* 22, e13146. doi: 10.1111/cmi.13146

Jain, B. P., and Pandey, S. (2018). WD40 Repeat Proteins: Signalling Scaffold With Diverse Functions. * Protein J.* 37, 391–406. doi: 10.1007/s10930-018-1975-7

Kariuki, M. M., Kaira, J. K., Mulaa, F. K., Mwangi, J. K., Wasunna, M. K., and Martin, S. K. (1998). Plasmodium falciparum: Purification of the Various Gametocyte Developmental Stages From *In Vitro-Cultivated* Parasites. *Am. J. Trop. Med. Hyg.* 59, 505–508.

Kaufish, M., Nehra, A., Gakhar, S. K., Gill, S. S., and Gill, R. (2020). The Multifaceted Histone Chaperone RbAp46/48 in *Plasmodium falciparum*: Structural Insights, Production, and Characterization. *Parasitol. Res.* 119, 1753–1765. doi: 10.1007/s00436-020-06669-5

Kuehn, A., and Pradel, G. (2010). The Coming-Out of Malaria Gametocytes. *J. Biomed. Biotechnol.* 2010, 976827. doi: 10.1155/2010/976827

Kuehn, A., Simon, A., and Pradel, G. (2010). Family Members Stick Together: Multi-Protein Complexes of Malaria Parasites. *Med. Microbiol. Immunol.* 199, 209–226. doi: 10.1007/s00281-010-0157-y

Kumar, N. (1987). Target Antigens of Malaria Transmission Blocking Immunity Exist as a Stable Membrane Bound Complex. *Parasitol. Immunol.* 9, 321–335. doi: 10.1111/j.1365-3024.1987.tb00511.x

Kumar, N., and Wikel, S. (1992). Further Characterization of Interactions Between Gamete Surface Antigens of Plasmodium falciparum. *Mol. Biochem. Parasitol.* 53, 113–120. doi: 10.1016/0166-6851(92)90013-x

Lamarque, M., Besteiro, S., Papoin, J., Roques, M., Vulliez-Le Normand, B., Wagner, C., Mejia, C., and Templeton, T. J. (2006). *Plasmodium falciparum* : Co-Dependent Expression and Co-Localization of the PfCCp Family of Adhesion Proteins. *J. Biol. Chem.* 281, 14357–14356. doi: 10.1074/jbc.M808472200

Miyawaki, P., Uthaipibull, C., Wong sombat, C., Kamchonwongpaisan, S., et al. (2004). A Multidomain Adhesion Protein Family Expressed in *Plasmodium falciparum* : Co-Dependent Expression and Co-Localization of the PCCp Multi-Adhesion Domain Proteins. *Exp. Parasitol.* 112, 263–268. doi: 10.1016/j.exppara.2005.11.010

Prompana, P., Utaiapibull, C., Wongsombat, C., Kamchonwongpaisan, S., Yuthavong, Y., Kneueper, E., et al. (2013). Inducible Knockdown of Plasmodium Gene Expression Using the glmS Ribozyme. *PloS One* 8, e73783. doi: 10.1371/journal.pone.0073783

Richard, D., MacRaid, C. A., Riglar, D. T., Chan, J. A., Foley, M., Baum, J., et al. (2010). Interaction Between Plasmodium falciparum Apical Membrane Antigen 1 and the Rhoptry Neck Protein Complex Defines a Key Step in the Exflagellate Invasion Process of Malaria Parasites. *J. Biol. Chem.* 285, 14815–14822. doi: 10.1074/jbc.M109.080770

Riglar, D. T., Richard, D., Wilson, D. W., Boyle, M. J., Dekiwadia, C., Turnbull, L., et al. (2011). Super-Resolution Dissection of Coordinated Events During Malaria Parasite Invasion of the Human Erythrocyte. *Cell Host Microbe* 9, 9–20. doi: 10.1016/j.chom.2010.12.003

Scholt, S. M., Simon, N., Lavazec, C., Dude, M. A., Templeton, T. J., and Pradel, G. (2008). PCCp Proteins of Plasmodium falciparum: Gametocyte-Specific Expression and Role in Complement-Mediated Inhibition of Exflagellation. *Int. J. Parasitol.* 38, 327–340. doi: 10.1016/j.ijpara.2007.08.009

Shen, B., and Sibley, L. D. (2012). The Moving Junction, a Key Portal to Host Cell Invasion by Apicomplexan Parasites. *Curr. Opin. Microbiol.* 15, 449–455. doi: 10.1016/j.mib.2012.02.007

Simon, N., Kuehn, A., Williamson, K. C., and Pradel, G. (2016). Adhesion Protein Complexes of Malaria Gametocytes Assemble Following Parasite Transmission to the Mosquito. *Parasitol. Immunol.* 65, 27–30. doi: 10.1016/j.paraitn.2015.09.007

Simirnmann, C. U., Potsalaki, E., Russel, R. B., and Müller, C. W. (2010). WD40 Repeat Proteins Promote Cellular Networks. *Trends Biochem. Sci.* 35, 565–574. doi: 10.1016/j.tibs.2010.04.003

Stirnimann, C. U., Petsalaki, E., Baumeister, S., Simon, N., Ngongang, V. N., Spehr, M., and Müller, C. W. (2010). WD40 Repeat Proteins Trigger Commitment to Invasion. *Proc. Natl. Acad. Sci. USA* 108, 13275–13280. doi: 10.1073/pnas.1100303108

Voon, L. V. D., and Ploegh, H. L. (1992). The WD-40 Repeat. *Nature Struct. Mol. Biol.* 9, 321–326. doi: 10.1038/nsmb.1998

Williamson, K. C., Keister, D. B., Muratova, O., and Kaslow, D. C. (1995). Recombinant PfP230, a *Plasmodium falciparum* Gametocyte Protein, Induces Antigens That Reduce the Infectivity of *Plasmodium falciparum* to Mosquitoes. *Mol. Biochem. Parasitol.* 73, 33–42. doi: 10.1016/0166-6851(95)80257-3

Wirth, C. C., Glushakova, S., Scheuermanny, M., Repnik, U., Garg, S., Schack, D., et al. (2014). Perforin-Like Protein PPLP2 Permeabilizes the Red Blood Cell Membrane During Egress of Plasmodium falciparum Gametocytes. *Cell Microbiol.* 16, 709–733. doi: 10.1111/cmi.12288

Xu, C., and Min, J. (2011). Structure and Function of WD40 Domain Proteins. *Protein Cell* 2, 202–214. doi: 10.1007/s13238-011-1018-1

Zhang, M., Wang, C., Otto, T. D., Oberstaller, J., Xiao, L., Adapa, S. R., et al. (2018). Uncovering the Essential Genome of the Human Malaria Parasite *Plasmodium falciparum* by Saturation Mutagenesis Science 360. doi: 10.1126/science.aap7847

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