Unravelling a PhIL1-glideosome associated complex in *Plasmodium falciparum* merozoite that is essential for invasion in host erythrocytes

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Research Article

Keywords: Malaria, *P. falciparum*, Inner Membrane Complex, Merozoite invasion, Glideosome

Posted Date: October 6th, 2020

DOI: <https://doi.org/10.21203/rs.3.rs-87875/v1>

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Abstract

The human malaria parasite, *Plasmodium falciparum* possess a unique mechanism of gliding motility guided by glideosome that powers its entry into insect and vertebrate hosts to facilitate its invasion and internalization within the targeted host cell. Photosensitized INA-labelled protein 1 (PhIL1) forms a novel protein complex that is associated with glideosome motor complex in the inner membrane complex of invasive merozoite. To establish the role of PfPhIL1 associated novel complex at asexual blood stages, we characterized three proteins associated with PhIL1: a glideosome associated protein- PfGAPM2, an IMC structural protein- PfALV5 and a previously uncharacterised protein - referred here as PfPhIP (PhIL1 interacting protein). GFP targeting and co-immunoprecipitation analysis confirmed that these proteins are part of a PhIL1 associated novel complex, which co-exists with the glideosomal complex. To know the functional significance of PhIL1 associated complex, transgenic parasites were generated for *glmS* mediated conditional knock-down of each of the three proteins. Parasites lacking PfPhIP or PfGAPM2 were unable to invade the RBCs. PfPhIP deficient parasites also showed defects in merozoite segmentation. PfPhIP and PfGAPM2 depleted parasites revealed abrogation of reorientation/gliding, although initial attachment with human RBCs was not affected in these knock-down parasites. Together, the data presented here shows that proteins of the PhIL1 associated complex play an important role in orientation of *P. falciparum* merozoites post initial attachment, which is crucial for formation of tight junction and hence invasion of host erythrocytes.

Introduction

The apicomplexan parasites invade different host cells such as erythrocytes, lymphocytes, macrophages, hepatic cell etc and their invasion is brought about by specific stages of each parasite referred as zoites. The zoites invade host cells by substrate dependent locomotion referred as gliding motility, which is powered by actomyosin motor; the glideosome (Boucher and Bosch, 2015) that resides within the pellicle attached to the inner membrane complex (IMC) lying underneath the plasma membrane of each zoites. The IMC is composed of flattened membrane cisternae or alveolar vesicles, whose cytoplasmic face is connected to subpellicular microtubules and a subpellicular protein network (SPN). The IMC of an *Apicomplexan* parasite plays diverse roles in maintaining structural stability of the zoite forms. It also acts as a scaffold for daughter cell development and plays a key role in motility and host-cell invasion. The SPN is composed of several putative structural alveolin (or IMC) proteins and IMC sub-compartment proteins (ISPs), that belong to broadly conserved protein families throughout Alveolate taxa (Gould et al., 2008; Woo et al., 2015). In addition to the structural components of the IMC, the actomyosin motor complex or the glideosome is a central component of the invasion and motility machinery. Proteins involved in the organization of pellicle/IMC/glideosome include structural proteins such as alveolins (IMC1a-h), glideosome associated protein- 40, -45 and -50 and glideosome associated proteins with multiple membrane spans (GAPMs), ISPs and these proteins together with MTIP (Myosin A tail domain interacting protein) anchor the actomyosin motor complex to the IMC (Daher and Soldati-Favre, 2009;

Poulin et al., 2013). These structures and proteins of glideosome/IMC are important as any disruption in assembly of IMC, blocks parasite invasion as well as sexual stage development (Parkyn Schneider et al., 2017).

To date, it is believed that the gliding or the motor complex is highly conserved among apicomplexans i.e. the same invasion motor supports all the different invasion pathways making it an exclusive target for designing new intervention strategies. Genetic manipulation/depletion of the genes encoding various core components of the motor complex for instance aldolase, myosin, MIC2, actin and GAP45, did not entirely abolish motility or host cell invasion by the zoites, implying that there might be alternative mechanisms for merozoite motility and invasion (Andenmatten et al., 2013; Egarter et al., 2014; Shen and Sibley, 2014).

Despite these known IMC-associated protein families and complexes, we have limited knowledge of how the shape, integrity and development of the IMC and cell pellicle are controlled. The underlying functions of the IMC and its core complexes are still unexplored. Photosensitized INA-Labelled protein 1 (PhIL1) was first identified in *Toxoplasma gondii* through Photosensitized labelling with 5-[¹²⁵I] Iodonaphthaline-I-azide (INA) as an apicomplexan conserved protein that associates with IMC and or underlying cytoskeleton and is concentrated at the apical end of the parasite (Barkhuff et al., 2011; Gilk et al., 2006). Deletion of the *phil1* gene in *Toxoplasma* showed only a mild growth phenotype, including a reduced but not abolished parasite load in animal models, and a subtle morphological change of slightly shorter and wider parasites (Barkhuff et al., 2011). No loss of parasite motility was seen, although the trajectory of 3D gliding patterns did change (Barkhuff et al., 2011; Leung et al., 2014). We and others using gene targeting approaches next showed that *Plasmodium* PhIL1 is required for both asexual and sexual stages of parasite development (Parkyn Schneider et al., 2017; Saini et al., 2017). Gene disruption of PhIL1 prevented the formation of transmissible mature gametocytes (Parkyn Schneider et al., 2017). Further, we showed that PfPhIL1 is associated with some known glideosome associated proteins along with few unique proteins (Saini et al., 2017). Analysis of PfPhIL1 interactome identified Alveolin (ALV5), glideosome associated proteins GAP50, GAPM1, -2 and -3 and few novel uncharacterized proteins such as PhIL1 interacting protein; PhIP (PF3D7_1310770), PF3D7_1355600, PF3D7_1431100 and PF3D7_1430880.

In the present study, we generated GFP tagged transgenic lines for three PhIL1 interacting proteins; a glideosome associated protein- PfGAPM2, an IMC structural protein- PfALV5 and a newly identified protein- referred here as PfPhIP (PhIL1 interacting protein); and investigated the function of PhIL1 associated novel complex by using conditional knockdown approaches for three selected components. Phenotypic analysis of parasites lacking PfGAPM2 and PfPhIP showed a role of these proteins in reorientation of *P. falciparum* merozoites that resulted in the disruption of invasion of human erythrocytes. The work thus identifies a novel PhIL1 associated complex and its role in reorientation of merozoites towards the host surface, a step essential for tight junction formation and subsequent invasion of merozoites into RBCs.

Results

***P. falciparum* ALV5, PhIP and GAPM2 proteins co-localise with PhIL1 in the Inner membrane complex**

We have previously shown the expression and localization of PhIL1 in the IMC of the *P. falciparum* parasite and its interaction with some of the components of glideosomal complex (Saini et al., 2017). To investigate the existence of PhIL1-associated novel IMC protein complex in *P. falciparum*, we selected three proteins: a conserved protein of unknown function (PF3D7_1310700), referred here as PhIL1 Interacting Protein (PhIP); Alveolin 5 (PF3D7_1003600 or IMC1c), an IMC structural protein; and GAPM2 (PF3D7_0423500), and expressed these as GFP-tagged fusion proteins transgenic parasite lines (Supplemental Figure S1A). Expression of fusion protein was confirmed by western blot analysis of lysate from the transgenic parasites using anti-GFP antibodies (Supplemental Figure S1B, C and D). GFP-specific antiserum recognised a band of ~60 kDa for ALV5-GFP, ~43 kDa for PhIP-GFP and ~70 kDa for GAPM2-GFP fusion protein. Transgenic parasites expressing ALV5-GFP showed peripheral localisation in the schizont stage and in merozoites (Figure 1A). Parasites expressing PhIP-GFP or GAPM2-GFP showed similar pattern of peripheral localization in the IMC, as shown for PfPhIL1 (Figure 1B and 1C). These proteins co-localised with PhIL1 in the IMC at schizont stage of the parasite with a Pearson's colocalization coefficient of more than 0.7 in an indirect immunofluorescence assay (Figure 1D).

Subsequently, to establish the identity of PhIL1 associated novel complex, we performed pull-down assays using GFP-Trap beads with the parasite extracts prepared from these transgenic lines. Immunoprecipitates were digested with trypsin and peptides were analysed by mass spectrometry to identify the interacting partners. The glideosomal proteins GAP50, glideosome associated proteins with multiple membrane spans (GAPMs) 1, -2 and -3, and alveolin/ IMC protein family were identified in each precipitate, together with PhIP (PF3D7_1310700) and ALV5 (Table 1). Overall, these results confirmed the interactions among these proteins as well as with the PhIL1 protein.

PhIL1 associated novel complex is closely associated with the glideosomal complex

To ascertain the association of PhIL1 associated protein complex with the glideosomal complex, we performed sedimentation analysis of *P. falciparum* 3D7 schizont/ merozoite lysate using glycerol density gradient centrifugation. Western blot analysis of the glycerol gradient fractions using anti-PfALV5, anti-PfPhIP, anti-PfGAPM2, anti-PfPhIL1, and anti-PfGAP50 antibodies; revealed that these proteins co-sedimented together in fractions 5 to 11, particularly in fraction 9 corresponding to ~250 kDa molecular mass (Figure 2A and supplemental figure S2), suggesting that these proteins probably are associated together in the parasite.

To further substantiate these results, schizont stage parasite lysate was subjected to blue native PAGE (BN-PAGE) followed by immunoblot analysis. As shown in figure 2B, we detected a high-molecular mass complex of ~800 kDa consisting of ALV5, PhIP, GAPM2, PhIL1 and GAP50. In addition, we observed another low molecular weight complex with anti-PhIL1 and anti-GAPM2 antibodies. Together, these results suggested that PhIL1 is associated with two different complexes; a high molecular weight

complex of ~800kDa consisting of components of PfPhIL1 associated complex and GAP50 and a low molecular weight complex of ~250kDa consisting of PhIL1 and GAPM2 (Figure 2B), indicating the heterogeneity among these two complexes, which are composed of different but overlapping proteins .

Taken together, results presented here (Figures 1 and 2) validate the association of these proteins with each other, and in particular, their interactions with select, but not all components of the glideosome machinery. The data thus illustrates that these proteins probably form an independent complex in the IMC, which may have a diverse role than the glideosome complex. Based on the above results, we propose that PhIL1 forms a novel complex probably in the outer IMC, having overlapping components with the glideosomal motility complex. The organisation of the proposed novel PhIL1 associated complex is depicted in Figure 2C.

PhIL1 associated complex plays an important role in parasite growth and invasion

To address the role of PfALV5, PfPhIP and PfGAPM2 proteins in the *P. falciparum* IMC, we generated conditional knock-down parasite lines expressing respective genes in fusion with HA-*glmS*. The *glmS* ribozyme is expressed downstream of the target gene, which is efficiently knocked down in response to glucosamine (GlcN). Strategy for generating the knock-down lines is presented in Supplemental Figure S3A. Integrates were enriched with blasticidin selection and cycling, followed by clonal selection of a single transgenic parasite by limiting dilution. Integration into the parasite genome was confirmed by PCR (Supplemental Figure S3B, C and D).

Expression and efficient knockdown of the fusion protein was analysed by western blot analysis of lysate from transgenic parasites using anti-HA antibody under the effect of GlcN inducer (Figure 3A, B and C inset). Ring stage parasites at 16-20 hpi were treated with GlcN (2.5 mM) and parasites were harvested at 42-44 hpi. The saponin lysed treated parasites were subjected to lysis in RIPA buffer followed by freeze-thaw cycles and the lysate was subjected to SDS-PAGE. PfBiP, a constitutively expressed endoplasmic reticulum chaperon protein was used as a loading control. Apparent knockdown of up to ~80-85% was achieved for the expression of ALV5-HA, PhIP-HA and GAPM2-HA in the respective parasite lysates.

To study the growth of knockdown parasites, GlcN was added at the ring stage parasites 16-20 hpi at varying concentrations (0.6 mM, 1.25 mM, 2.5 mM, and 5 mM) and the growth was monitored till the formation of new rings i.e. up till one invasion cycle. Loss of ALV5 had little effect in the invasion of human RBCs by merozoites in comparison to the wild type parasites (Figure 3A), however PhIP depleted parasites showed ~80% invasion inhibition at 5 mM glucosamine (Figure 3C) while reduction in GAPM2 levels exhibited an invasion inhibitory potential of ~80% at 1.25 mM concentration of GlcN (Figure 3E).

Representative Giemsa stained smears of the ALV5 knockdown parasites showed no significant difference apart from a slightly delayed parasite growth cycle in the GlcN treated (5 mM) and untreated parasites, suggesting that ALV5 is not essential for parasite growth (Figure 3B). In comparison, the PhIP-HA-*glmS* parasites showed arrested development of schizonts in the knockdown line (1.25 mM GlcN treatment) (Figure 3D). Following PhIP knockdown, a proportion of schizonts phenotypically displayed

incomplete segmentation showing agglomerates of unsegmented merozoites in close proximity to the food vacuole, which might be due to failure of IMC formation. Some of the parasites, which were fully segmented and merozoites egressed normally, however, these merozoites were unable to invade and newly released merozoites were seen arrested on the surface of RBC suggesting that, despite the initial attachment, parasite was unable to penetrate into the host RBC. In PhIP knockdown parasites two distinct parasites were observed, ~43% showed unsegmented merozoites, while 57% of the PhIP depleted parasites were found to be arrested at erythrocyte surface after attachment (Figure 3D-zoom and supplemental figure S4). In the absence of GlcN, distinct merozoites were observed enclosed in schizonts and these merozoites invaded normally as seen with their ability to progress to ring stage (Figure 3D). By contrast, treatment of GAPM2-HA-*gImS* with GlcN resulted in normal merozoite egress, however released merozoites were found to be stuck at erythrocyte surface, indicative of their inability to invade the RBC. However, we did not observe any defect in the attachment of these merozoites to RBC surface (Figure 3F).

PfPhIP knock-down causes underdeveloped IMC/plasma membrane during schizogony

We further studied the defects in merozoite segmentation in PfPhIP knock-down parasites for the formation of the parasite plasma membrane (PPM), formation of and secretion by apical secretory organelles and IMC formation by immunostaining these parasites with either with anti-GAP50, or anti-MSP1, or anti-AMA1 antibodies. Briefly, schizonts maintained with and without GlcN from the early ring-stage were treated with 10 mM E64 at 42 hpi, to prevent release of daughter merozoites. In PhIP knockdown parasites, multiple daughter cells remained partially attached to each other. In these parasites, we were able to observe residual signal for PhIP as the efficiency of knockdown was ~80%, not the complete knockout. In PfPhIP-knockdown parasites, segmented merozoites showed detectable PfPhIP staining by IFA, whereas in the residual unsegmented agglomerate PfPhIP staining was not detected (Figure 4A). PhIP depleted parasites showed apparent loss of signal for GAP50 in the multi-nucleated agglomerates suggesting defect in IMC formation in agglomerates while [-] GlcN parasites, showed well-formed IMC around each nucleus of the segmented schizont (Figure 4B). Parasite plasma membrane which coats the individual newly formed daughter cells was examined by Merozoite Surface Protein 1 (MSP1). PhIP depleted schizonts showed MSP1 staining enclosing multiple nuclei of the agglomerate inside one contiguous membrane in contrast to untreated parasites that showed MSP1 surrounding each segmented daughter merozoite nucleus discretely (Figure 4C and Supplemental Figure S5A). Thus, in the agglomerates, PhIP knock-down parasites failed to direct the PPM around single daughter nuclei. Simultaneously, microneme formation and secretion was assessed using anti-PfAMA1 (Apical Membrane Antigen 1) antibody in [-] GlcN as well as [+] GlcN parasites. [-] GlcN PhIP-HA-*gImS* parasites showed AMA1 staining around each merozoite, whereas PhIP depleted parasites demonstrated surface AMA1 staining in fully segmented merozoites, whereas loss of AMA1 signal on merozoite surface was observed for the multi-nucleated agglomerates (Figure 4D and Supplemental Figure S5B). 3D reconstruction of schizont stage transgenic parasites with and without GlcN with respect to different marker antibodies is illustrated using Imaris, version 7.6.1 (Bitplane) (Figure 4E).

These results established that PfPhIP-deficient parasites show developmental defect during the final stages of schizont segmentation that fail to reinstate the asexual blood cycle due to structural defects.

PfPhIP and PfGAPM2 knock down results in generation of non-invasive merozoites defective in apical organelle secretion

Since we observed that merozoites in PfPhIP and GAPM2 knock-down parasites were able to attach to the RBC surface, but could not invade, we next performed immunostaining of these RBC attached but non-invasive merozoites, to determine whether the inability of merozoites to invade the RBCs is due to defects in apical organelles biogenesis or their secretion, which is crucial for formation of invasion complex or due to the inability of motility complex which fails to propel the invading merozoite into the host RBC. To understand whether the IMC formation is affected in these knock-down parasites, we stained these attached merozoites with anti-PfGAP50 antibody. Importantly, PhIP deficient merozoites showed loss of signal for GAP50 suggesting defects in IMC formation (Figure 5A; panel 1) while GAPM2 depleted merozoites displayed intact IMC encircling the nascent attached cell (Figure 5B; panel 1). To assesses the merozoites released from the PhIP or GAPM2 deficient schizonts for the formation and secretion of apical organelles, we used α -EBA175, and α AMA-1 antibodies as markers for micronemes, and α -PfRON2 antibody as a rhoptry marker. We observed typical micronememal staining as visualized by the presence discrete dots with anti-EBA175 and anti-AMA1 antibodies. However, we could not locate these proteins on merozoite surface in the merozoites released from the PhIP and GAPM2 knockdown schizonts, therefore indicating the failure to discharge the micronemal contents post egress (Figure 5A and B; panel 2 and 3). We additionally evaluated the presence of PfRON2, a rhoptry marker in merozoites in PfPhIP- and PfGAPM2-knockdown parasites. These knock-down parasites displayed characteristic rhoptry localisation (Figure 5A and B; panel 3). Together, these results indicated that merozoites formed and released in PfPhIP- and PfGAPM2-knockdown parasites looked morphologically normal as indicated by their staining with the panel of cell markers. However, apical organelle secretion of the invasion ligands seems to be affected in the merozoites released from schizonts with PfPhIP and PfGAPM2 deficiency (Supplemental Figure S6A and B). 3D reconstruction of stuck merozoites from transgenic parasites with GlcN with respect to different marker antibodies are illustrated using Imaris, version 7.6.1 (Bitplane) (Figure 5C).

Since, the secretion of EBA175, AMA1, and RON2, failed to initiate following merozoite attachment in PfPhIP- and PfGAPM2-deficient merozoites, it clearly suggested that the signal for invasion in these merozoites post attachment to the erythrocytes was not triggered in PfPhIP- and PfGAPM2-knockdown parasites. On detailed analysis, we observed ~87% of the attached merozoites failed to align their apical end towards the erythrocyte surface i.e. the apex of the merozoite is not in direct proximity of the erythrocyte surface as indicated by staining with apical marker proteins. Apical reorientation is imperative for triggering commitment to invasion. Taken together, the analysis of attached PfPhIP- and PfGAPM2-deficient merozoites suggested roles of PfPhIP and GAPM2 in the reorientation of merozoites so that the apical organelles are aligned to the erythrocyte membrane (Figure 6). This study also highlights that

merozoite reorientation might directly or indirectly mediated by the motor complex due to existence of the overlapping components among these two complexes.

Discussion

A unifying feature of apicomplexan parasites is the presence of a surface pellicle and highly polarised cellular organisation with the apical complex at the anterior end of their invasive stages, which is implicated in motility and invasion of host cells/tissue (Morrisette and Sibley, 2002). The pellicle is composed of the parasite plasma membrane, the inner membrane complex (IMC) and a subpellicular network (SPN) that underlies the IMC. IMC acts as an anchor for motor complex, playing significant role in gliding and thus invasion (Boucher and Bosch, 2015; Frenal et al., 2010). Motility in the invasive stages is powered by an actomyosin motor termed as the glideosome, which resides within the pellicle located directly beneath the surface membrane and is tethered to the IMC. A number of studies have identified several proteins associated with the IMC and have provided insights into the organisation and roles of the glideosome complex. (Boucher and Bosch, 2015; Harding et al., 2016; Harding and Meissner, 2014; Jacot et al., 2016). The basic motor complex in apicomplexan parasites that drives gliding motility and invasion is comprised of conserved components such as actin-MyoA-MTIP-GAP45-GAP40-GAP50-GAPMs-aldolase. IMC has also been implicated to be involved in cell division (Harding and Meissner, 2014).

Earlier, we and others have shown *Plasmodium* PhIL1 to be localised in parasite IMC and its association with alveolins and other protein components that overlap with those in the known glideosome complex, including GAP50 (Parkyn Schneider et al., 2017; Saini et al., 2017). In the present study, we show the existence of PhIL1 associated complex and delineate the functional relevance of PhIL1 associated novel proteins in the *P. falciparum* merozoite. We selected three proteins from PhIL1 interactome namely, IMC1c or ALV5, a structural constituent of the SPN; a previously uncharacterised protein, PF3D7_1310700, which we termed PhIL1 Interacting Protein (PhIP); and GAPM2, a well-established component of the glideosomal complex. GFP tagged proteins of PhIP, ALV5 and GAPM2 confirmed their localisation to the IMC and co-localisation studies using indirect immunofluorescence assay affirmed the close association of these proteins with PhIL1 in asexual blood stage of the parasite. The alliance amongst these proteins were also established by reciprocal co-precipitation studies using GFP-Trap wherein GFP tagged proteins from all the 3 parasite lines (ALV5-, PhIP- and GAPM2-GFP) were co-precipitated with their interacting protein partners and components of the PhIL1 associated complex.

To know whether these proteins exists in a complex, we performed co-sedimentation and blue native page analysis of schizont stage parasite extracts. Although both these analysis suggested the existence of PhIL1 and its associated proteins in complex(s), however western blot analysis of Blue native page using their respective antibodies identified two discrete bands; a low molecular mass band corresponding to ~250kDa having PhIL1 and GAPM2 proteins and a high molecular weight band of ~800kDa consisting of ALV5, PhIP, GAPM2, PhIL1 and GAP50 in the IMC along with other components of the glideosomal complex, indicating the heterogeneity among these two complexes, which are composed of different but overlapping proteins. These results suggested a possible association of PhIL1 associated complex with

the glideosomal complex. It is conceived that the motor complex assembles at the N-terminus of the GAP50 anchor (Baum et al., 2006). It is possible that GAPM1 to -3 proteins, which span both the outer and inner side of the IMC, may be part of different complexes on either side of the IMC. Therefore, we speculate that PhIL1 associates with GAP50 at the other end of the IMC i.e. at its C-terminus along with GAPMs and alveolins (Bullen et al., 2009) and is a part of a novel complex at the inner IMC. Since GAP50 is both a luminal and transmembrane protein, it is possible that PhIL1 associated complex interacts with GAP50 on the inner membrane of the IMC and GAP50 interacts on the outer side with the myosin machinery like GAP45, GAP40, MyoA and MTIP.

We next characterised these selected components of the PhIL1 associated complex: ALV5, PhIP and GAPM2, using conditional knock-down approaches. *Plasmodium* alveolins are thought to be involved in parasite motility through interactions with the pellicular membrane embedded glideosomal components, apart from their role in morphogenesis and providing tensile strength. The conditional knockdown of ALV5 using *glmS* ribozyme slightly delayed the developmental time span at late stages of the asexual cycle, although the parasite could complete its growth leading to the formation of new rings. This could be due to the redundancy among 13 members of the alveolin family. These results are in line with a previous *PbIMC1d* knock-out study where no apparent phenotype was observed (Al-Khattaf et al., 2015). Even a double knockout of alveolins (IMC1b and IMC1h) revealed decreased tensile strength of ookinetes without affecting their morphology (Trempe and Dessens, 2011). It appears that glideosomal associated protein complexes exhibit considerable plasticity in their functions to ensure survival of the parasite. MyoA is reported to be recruited at the apical cap of the parasite through interactions with GAP45 or its paralog GAP70. Another member of this family, GAP80 recruits and assembles a new glideosome with MyoC at the basal polar region. Both these complexes share GAP50, GAP40 and MTIP. It was found that the deletion of MyoA is compensated by MyoC as it relocates to the apical end to initiate invasion and vice-versa (Frenal et al., 2014). All these studies uncover the fact that until now what was considered to be as a highly conserved machinery exhibits different protein complexes sharing some common components and displays complementary and compensatory mechanisms for successful invasion. These studies emphasize on high degree of complexity and functional versatility of the IMC components involved in gliding. Similar compensatory mechanisms have been reported for GAP45 and its ortholog at the basal region (Frenal et al., 2010). Recent investigations have demonstrated that parasite can invade the host cell in the absence of several core components of the glideosomal machinery (such as MyoA, MIC2, MLC1, GAP45 and actin), indicating the existence of an alternative motor mechanisms for invasion.

In contrast to the knock-down results for ALV5 parasites, PhIP and GAPM2 knock-down parasites showed pronounced effect on parasite invasion. Following PhIP knockdown, a proportion of schizonts displayed incomplete segmentation and multiple merozoites remained attached to each other, while distinct merozoites were observed enclosed in schizonts in wild type parasites. This resultant phenotype might be due to the failure of IMC biogenesis and stabilisation. Similar phenotype had been observed in case of Merozoite Organising Protein (PfMOP) knock-down parasites, where agglomerates were noted due to flawed segmentation (Absalon et al., 2016). Also, this is in agreement with PfSortilin knockdown study that showed involvement of Sortilin in IMC biogenesis (Hallee et al., 2018). Together, the data suggested

a possible role of PfPhIP in IMC formation in maturing schizonts and failure to do so results in the absence of plasma membrane enclosing individual daughter merozoites in the agglomerates. Similar observations have been reported upon depletion of GAP40 and GAP50, which resulted in defective IMC biogenesis and stabilisation during replication (Harding et al., 2016).

Some of the PhIP depleted schizonts were fully segmented and generated merozoites which egressed normally, however the merozoites released from these schizonts unable to invade host RBCs and got arrested on the surface of RBCs. These results suggest that, despite the initial attachment, merozoites were unable to penetrate into the host RBC, which might be due to a defective motor machinery leading to impaired reorientation of the invading merozoites. Recent work has provided evidence that specific proteins of the IMC can independently be involved in both motility and maintenance of cell shape and strength (Trempe and Dessens, 2011). The two distinct phenotypes observed due to ablation of PhIP, demonstrates the divergent functions of the components of IMC during asexual lifecycle of the parasite. We speculate that it might play a dual role in maintaining the cellular integrity of the daughter cells during cell division along with its role in reorientation of merozoites during the invasion process.

The conditional knock down of GAPM2-HA-*glmS* parasites showed the inability of merozoites to invade the RBCs. For GAPM2 conditional knock down parasites, we observed merozoites stuck on erythrocyte surface, indicative of their inability to invade the RBC. However, it was observed that the attachment of invasive merozoite is not affected in these parasites. While, untreated GAPM2-HA-*glmS* parasites progressed to ring stage infection. A recent study highlighted the role of GAPM1 and GAPM3 in providing the bridge between the sub-pellicular network and the alveoli in the IMC in order to maintain parasite structure and rigidity. GAPM1 depletion resulted in depolymerization of microtubules compromising parasite shape and integrity (Harding et al., 2019). However, there has been no report suggesting the role of GAPMs in invasion of the host.

Merozoite invasion is a complex, multistep process. First, there is a reversible attachment of merozoite to the erythrocyte surface through any part of the merozoite surface i.e. the apex of the merozoite is not in direct proximity of the erythrocyte surface followed by its apical reorientation so that the apical organelles are aligned to the erythrocyte membrane, formation of an irreversible tight junction (primarily involving AMA1 and RON) and ultimately its entry into the host cell powered through the motor complex. These steps are timed by organelle secretion and various signalling events (Cowman et al., 2012). To get insight into the defects in invasion of merozoites released from PfPhIP and PfGAPM2 knockdown schizonts, we assessed the expression and secretion of the invasion ligands using EBA175, AMA1 and RON2 antibodies. Despite showing expected expression pattern for the apical organelles, the release of these invasion ligands onto the merozoite surface appeared to be affected. These defects in organelle secretion were found to be due to failure of the attached merozoites to reorient their apical end towards the RBC surface; which in turn triggers the signaling events for the release of contents from apical organelles. The specific molecular interactions and mechanisms involved in apical reorientation are poorly understood. Till date, there is no evidence for the involvement of Glideosome or its associated motor protein complexes in mediating the reorientation of apical organelles of the merozoites towards

the host surface. A study (bioRxiv Preprint) recently identified the gliding ability of *Plasmodium* merozoites (Kazuhide Yahata, 2020). Data presented here suggests the role of PfPhIP and PfGAPM2 in the reorientation which might be directly or indirectly mediated through motor complex in *Plasmodium falciparum* merozoites.

In Conclusion, we have characterised *P. falciparum* ALV5; PhIP, a previously uncharacterised protein; and GAPM2. Conditional knockdown studies performed with these proteins showed that GAPM2 and PhIP are essential for the blood stage infection and their genetic attenuation arrests merozoite invasion by impeding the function of glideosomal motor machinery resulting in failure of merozoite to reorient its apical end towards the host RBC (Figure 6). Taken together, our results suggest that PhIL1 associated IMC complex is different in composition with that of previously described glideosomal complex and it appears to be essential for parasite invasion of erythrocytes. The study thus provides new molecular and mechanistic insights into contribution of IMC which will likely be effective in identifying new molecules for intervention strategies for malaria parasite development.

Experimental Procedures

Maintenance of *P. falciparum* cultures

P. falciparum parasite line 3D7 was maintained in O⁺ human erythrocytes (RBC) at 3% hematocrit in RPMI 1640 medium (pH 7.4) supplemented with 50 µg/ml hypoxanthine, 0.5% albumax II, 2 mg/ml sodium bicarbonate, and 20 µg/ml gentamycin. Cultures were incubated in air tight boxes at 37 °C in an atmosphere of 1% O₂, 4% CO₂ and 95% N₂ (Trager and Jensen, 2005).

Parasites were synchronized using sorbitol treatment (Lambros and Vanderberg, 1979). Briefly, the culture was harvested at about 10 % parasitemia with majority of parasites at the ring stage by centrifuging at 2000 rpm for 5 min at RT. To the cell pellet, 5 volumes of 5 % sorbitol solution was added and mixed gently. This solution was incubated at 37°C for 10 min and centrifuged at 2000 rpm for 5 min. The supernatant was carefully discarded without disturbing the pellet. Pellet was washed with prewarmed complete media twice and the culture was incubated at 37°C for the growth of the parasite.

P. falciparum parasite transfection

To generate a GFP-tagged transfection vector construct, the entire open reading frame of PfPhIP, PfALV5, and PfGAPM2 was amplified using gene specific primers (Supplemental Table 1), and cloned into the transfection vector pSSPF2 (Sato et al., 2003) at the *Bgl*II and *Avr*II restriction sites to create a fusion of desired gene of interest (GOI) with green fluorescence protein (GFP) under the control of the *hsp86* promoter. Synchronized *P. falciparum* 3D7 ring stage parasites were transfected with 100 µg of purified plasmid DNA (Plasmid Midi Kit, RBC) by electroporation (310 V, 950 µF) (Crabb et al., 2004) and the transfected parasites were selected using 2.5 nM blasticidin. Transfected parasites were detectable after one to two months following transfection. To detect expression of the PfGOI-GFP fusion protein in the

transgenic line, parasite lysates were analysed using 12% SDS-PAGE and western blotting with anti-GFP antibody.

For the generation of knock-down constructs, C-terminal region of PfPhIP, PfALV5, and PfGAPM2 was amplified using gene specific primers (Supplemental Table S1), and cloned into the transfection vector pHA-glmS (Elsworth et al., 2014) using *Pst*I and *Bgl*II restriction sites to create a fusion of desired gene of interest (GOI) with HA-glmS under the control of native promoter. The ring stage parasites were transfected as mentioned and transgenic parasites were selected on alternate blasticidin drug ON and OFF cycles to ensure genomic integration of PfGOI-HA-glmS constructs. The transgenic parasites were then subjected to clonal selection by serial dilution to obtain parasite line from a single genome integrated clone.

Isolation of parasites, extraction of proteins and immunoblotting

Expression of the PfGOI-GFP or PfGOI-HA fusion protein in transgenic *P. falciparum* blood stage parasites was examined by western blotting. Briefly, schizont stage parasites were isolated following lysis of infected erythrocytes with 0.15% saponin; following centrifugation the pellet was resuspended in RIPA buffer (150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris pH 8.0) and cells were lysed at 4°C for 30 min, followed by 3 cycles of freeze-thaw in liquid N₂ and at 37°C. A clear parasite lysate was obtained by centrifugation at 13,000 rpm for 30 min at 4°C. The supernatant was then mixed with Laemmli buffer, boiled and centrifuged, and proteins were separated on 12% SDS-PAGE. The fractionated proteins were transferred from the gel on to the PVDF membrane (Millipore) for 2 h at 200 mA and then the membrane was treated with blocking buffer (5% milk powder in 1 × PBS) overnight at 4°C. The blot was washed and incubated for 1 h with rabbit anti-GFP (1: 10,000) or rat anti-HA (1:1000), diluted in dilution buffer (1 × PBS, 0.1% Tween-20, and 1% milk powder). The blot was subsequently washed with PBS containing Tween-20 (PBST) and incubated for 1 h with secondary anti-rabbit/anti-rat IgG antibody (1: 300,000) conjugated to HRP. Protein bands were visualized using an ECL detection kit (Thermo Scientific, USA).

Fluorescence microscopy

To visualize GFP expression, the transgenic parasite suspension was incubated with DAPI (2 ng/ml) in PBS at RT for 10 min. Following three washes with 1 × PBS (pH 7.4); samples were mounted on glass slides and observed on a Nikon A1 Confocal Microscope (Nikon Corporation, Tokyo, Japan).

Indirect Immunofluorescence Assay

Parasites were fixed with fixation solution containing 4% paraformaldehyde and 0.0075% glutaraldehyde in PBS for 30 min. After washing with PBS, parasites were subjected to permeabilization with 0.1% Triton X-100 for 15 min followed by blocking with 10% FBS in PBS for 2 h. After blocking, parasites were probed with primary antibody for 1 h, followed by secondary antibody for 1 h at RT. The parasites were washed and incubated with DAPI to stain the nucleus for 10 min at RT and imaged using a Nikon A1-R confocal

microscope (Nikon Corporation, Tokyo, Japan). The images were analysed by NIS elements software (Nikon). The antibody combinations used for various experiments are: 1. For colocalization with the GFP line, rabbit α -PfPhIL1 (1:100) was used, 2. For the colocalization studies in knockdown experiments, rabbit α -PfGAP50 (1:100), rabbit anti-PfMSP1 (1:250), rabbit α -PfAMA1 (1:100), rabbit α -PfEBA175 (1:100), mice α -PfRON2 (1:50) and rat α -HA (1:100) were used; followed by appropriate secondary antibodies: anti-mice Alexafluor 488 (1:500), anti-rabbit Alexafluor 488 (1:500), anti-rabbit Alexafluor 594 (1:500), and anti-rat Alexafluor 488 (1:500) (Invitrogen). Nucleus was stained using DAPI.

Conditional knock down assay

The functional role of PfALV5, PfPhIP and PfGAPM2 was determined by inducible knock down with glucosamine. Effect of knock down on parasite invasion was evaluated w.r.t 3D7 strain of *P. falciparum*. The parasite lines (PfGOI-HA-glmS transgenic and 3D7) were synchronized using 5% sorbitol and the assay was set at the mid ring stage with a haematocrit and parasitaemia of synchronized ring stage culture adjusted to 2% and 1%, respectively. Glucosamine was added to the parasite culture at varying concentrations (0.3 mM, 0.6 mM, 1.25 mM, 2.5 mM, and 5 mM). Parasite growth was monitored microscopically after every 8 h by Giemsa stained smears. The parasitaemia was estimated after an incubation of 40 h in the next cycle using flow cytometry. Briefly, cells from samples were collected and washed with PBS followed by staining with ethidium bromide (10 μ g/ml) for 20 min at 37°C. The cells were subsequently washed twice with PBS and analysed on FACSCalibur (Becton Dickinson) using the Cell Quest Pro software. Fluorescence signal (FL2) was detected with the 590 nm band pass filter using an excitation laser of 488nm collecting 100000 cells per sample. Uninfected RBCs stained in similar manner were used as control. Following acquisition, data were analysed for percentage parasitaemia of each sample by determining the proportion of FL2-positive cells using Cell Quest and percent inhibition was calculated relative to the GlcN untreated PfGOI-HA-glmS parasite cultures.

Co-precipitation of interacting proteins

For Pull-down of GFP-fusion proteins, schizont stage lysate was obtained as described above and immunoprecipitation was done using GFP-Trap[®]_A Kit (Chromotek) following the manufacturer's instructions. GFP-Trap[®]_A beads were equilibrated with dilution buffer and allowed to bind to proteins in the parasite lysate by tumbling the tube end-over-end for 3 h at 4°C. Samples were then centrifuged at 1600 rpm for 1 min and the beads washed twice with dilution buffer. Proteins were eluted in 50 μ l elution buffer and peptides were analysed by mass spectrometry following in-solution digestion.

Glycerol Density Gradient fractionation for isolation of complexes

The sedimentation curve of molecular mass standards in different fractions and the protocol followed is as described earlier (Chugh et al., 2013). Briefly, schizont stage parasites were lysed in as described above. Lysate was cleared by centrifugation, and 500 μ l of lysate was layered on top of a 9 mL 5-45% glycerol step-gradient (45% glycerol solution being the lowermost and 5% glycerol solution being the topmost layer). Gradients were centrifuged at 38,000 rpm for 18 h at 4°C in a SW41 Ti rotor (Beckman).

Twenty, 0.5 mL fractions were collected from each gradient, and equal volumes of each fraction was mixed with sample loading dye. Alternate protein fractions were resolved by SDS/PAGE and analysed by Western blotting using protein specific antibodies at 1:5000 dilution for GAP50, PhIL1, ALV5, PhIP and GAPM2 followed by secondary α -rabbit antibody at 1:100000 dilution and detection using an ECL detection kit (Thermo Scientific, USA).

Native gel electrophoresis

BN-PAGE analysis was carried out to separate various complexes present in the invasion supernatant using NativePAGE™ Novex® Bis-Tris Gel System (Life technologies) following manufacturer's protocol. Briefly, Schizont stage parasite lysate was mixed with NativePAGE sample buffer, and NativePAGE 5% G-250 sample additive, and resolved in the NativePAGE™ Novex® 4–16% Bis-Tris Gels at 4°C, which resolve proteins in the molecular weight range of 15-1,000 kDa and then transferred to polyvinylidene difluoride (PVDF) membranes. To identify PfPhIL1 associated complexes, membrane were probed with rabbit anti-PfGAP50, anti-PfPhIL1, anti-PfALV5, anti-PfPhIP and anti-PfGAPM2 (1:5000 dilution each) antisera, followed by incubation with HRP-conjugated goat anti-rabbit IgG secondary antibody (1:100,000).

Declarations

Author Contributions: Conceptualization, P.M., A.M., and E.S.; Writing – P.M., A.M., and E.S.; Intellectual input, illustrations, and scientific discussions, P.M., A.M., E.S. and P.K.; E.S., P.K., and V.S. conducted the experiments; I.K. performed the mass spectroscopy; Funding Acquisition, P.M., and A.M.

Acknowledgements: ES acknowledges the financial support from Department of Biotechnology, Government of India in the form of DBT-JRF Fellowship. PS and VS are awardees of fellowship from University Grants Commission (UGC) and Council of Scientific & Industrial Research (CSIR), Govt. of India, respectively. We thank Rotary blood bank, for providing packed red blood cells. We thank Dr. Chetan E. Chitnis and Dr. Mansoor A Siddiqui for providing PfAMA1 and PfEBA175 antibodies. We are grateful to Paul Gilson for providing vector pHA-*gImS* and Siggie Sato for pSSPF2 vector. The research work in PM's and AM's laboratory is supported by Flagship Project (BT/IC-06/003/91), Indo-Danish grant (BT/PR5267/MED/15/87/2012) and Centre Of Excellence grant (BT/COE/34/SP15138/2015) from Department of Biotechnology, Govt. of India. The funders had no role in the design of the study and collection, analysis, and interpretation of data and in writing the manuscript.

Disclosure of Interest: The authors declare no competing interests.

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Tables

Table 1 List of proteins pulled down by GFP-Trap beads from lysates of ALV5, PhIP and GAPM2-GFP parasites, respectively. n = 3 experiments.

Gene ID	Gene product	# unique peptides identified by GFP-TRAP pull down		
		ALV5	PhIP	GAPM2
PF3D7_1003600	Membrane skeletal protein IMC1-related (ALV5)	12	4	3
PF3D7_0525800	Membrane skeletal protein IMC1-related	14	5	4
PF3D7_0918000	Secreted acid phosphatase (GAP50)	7	4	14
PF3D7_0822900	Conserved Plasmodium protein, unknown function	8	-	6
PF3D7_1310700	Conserved Plasmodium protein, unknown function	2	3	2
PF3D7_0109000	Photosensitized INA-labeled protein 1, PhIL1, putative	4	-	4
PF3D7_1406800	Glideosome associated protein with multiple membrane spans 3 (GAPM3)	4	-	5
PF3D7_0423500	Glideosome associated protein with multiple membrane spans 2 (GAPM2)	3	-	4
PF3D7_1323700	Glideosome associated protein with multiple membrane spans 1 (GAPM1)	1	-	-
PF3D7_1246400	Myosin light chain 1, myosin A tail domain interacting protein (MTIP)	1	-	-
PF3D7_1222700	Glideosome-associated protein 45 (GAP45)	1	-	1
PF3D7_0304000	Inner membrane complex protein 1a, putative (IMC1a)	1	-	-
PF3D7_0613900	Myosin E, putative (MyoE)	1	-	2
PF3D7_1342600	Myosin A (MyoA)	-	1	1

Figures

Figure 1

PhIL1 co-exists with ALV5, PHIP, and GAPM2 in the *P. falciparum* IMC: (A) ALV5-GFP expression pattern in asexual blood stages (merozoite, ring, trophozoite and schizont) of *P. falciparum*. (B) PHIP-GFP expression pattern in asexual blood stages (merozoite, ring, trophozoite and schizont) showed typical IMC localisation. (C) GAPM2-GFP expression pattern in asexual blood stages. (D) Co-staining of PhIL1 with ALV5, PHIP and GAPM2 in *P. falciparum* blood-stage schizont. These proteins co-localised with

PhIL1 in the IMC at schizont stage of the parasite with a Pearson's colocalization coefficient of more than 0.7. Scale bar = 5 μ m.

Figure 2

PhIL1 associated novel complex overlaps with components of the linear motor in the IMC but is a separate complex: (A) PhIL1 associated complex co-exists with the glideosomal complex as seen by Glycerol gradient co-sedimentation analysis. Glycerol gradient fractionation of *P. falciparum* schizont extract using 5 to 45% Glycerol gradient followed by immunoblotting of the fractions using specific antibodies showed the co-sedimentation of PfGAP50, PfPhIL1, PfALV5, PfPhIP and PfGAPM2 together in fractions 5 to 11, particularly, in fraction 9 corresponding to ~250 kDa molecular mass. $n = 2$ experiments. (B) Native PAGE analysis of schizont stage parasite lysate shows co-existence of PhIL1 associated complex with the glideosomal complex via. some overlapping components. A complex of ~800 kDa was detected containing components of PfPhIL1 associated complex and GAP50. Furthermore, a smaller complex of ~250 kDa was also detected that included PfPhIL1 and PfGAPM2. These results indicate a possible association of PhIL1 associated complex and glideosomal complex. $n = 2$ experiments. (C) Prototype showing molecular motor and partially overlying proposed PhIL1 complex in the parasite IMC. Photosensitized 5-[125I] Iodonaphthalene-1-azide Labelled Protein-1 (PhIL1); PhIL1 Interacting Protein (PhIP); Glideosome-associated protein 40, 45 and 50 (GAP40, 45 and 50); Glideosome-associated protein with multiple membrane spans (GAPM); myosin-A (MyoA); myosin-A tail domain interacting protein (MTIP); Glideosome Associated Connector (GAC); Protein of the Merozoite surface (MzP); Parasite Plasma Membrane (PPM).

Figure 3

Functional characterization of *P. falciparum* ALV5, PhIP and GAPM2 by inducible regulation of endogenous protein levels and their effect on parasite growth: (A) Effect of conditional knockdown of PfALV5 on parasite invasion of the host RBC. Glucosamine-treated (+GlcN, 2.5 mM) and untreated (-GlcN) cultures were incubated till the formation of new rings and the parasitaemia was estimated by flow cytometry. Inset: Western blot analysis of lysate from PfALV5-pHA-glmS line with α -HA rat serum showing conditional knock-down on glucosamine treatment. (B) Representative parasites from the Giemsa stained smears showing morphology following ALV5 knockdown. (C) Effect of conditional knockdown of PfPhIP in parasite. Inset: Western blot analysis of lysate from PfPhIP-pHA-glmS line with α -HA rat antibody to check for conditional knock-down shows a robust knockdown of PhIP-HA protein. (D) Representative parasites from the Giemsa stained smears showing morphology following PhIP knockdown. Percentage

of different phenotypes was calculated from Giemsa smears of glucosamine treated PhIP-HA-glms parasites, highlighting the arrest of growth in segmented schizont stage, and altered efficacy of released merozoites to invade following PhIP knockdown. See also Figure S4. (E) Effect of conditional knockdown of PfGAPM2 in parasite showing upto 85% invasion inhibition. Inset: Western blot analysis of lysate from PfGAPM2- HA-glms line with α -HA rat serum showing efficient knockdown of the GAPM2-HA protein when compared to the anti-PfBip loading control. (F) Representative Giemsa-stained smears showing the arrest in development following GAPM2 knockdown due to inefficiency of released merozoites to invade the host RBC. Zoomed Giemsa smear show phenotype for PhIP- and GAPM2-HA-glms parasites plus glucosamine, highlighting the arrest in development following knockdown. Data represent mean $\pm$ SD. n = 3 experiments. Anti-PfBip was used as loading control for western blot analysis.

Figure 4

PfPhIP deficient parasites show defects in segmentation of daughter cells: PfPhIP-HA-glms parasites maintained with and without GlcN were E64-treated (10 mM) and probed with the anti-HA antibody (green) and counterstained with DAPI. (A) Apparent knockdown of PhIP expression leads to formation of agglomerates of unsegmented daughter nuclei. (B) Anti-GAP50 antibody (IMC marker) (red) showed defects in IMC formation in agglomerates. Merozoite plasma membrane and micronemes were stained with antibodies against *P. falciparum* (C) merozoite surface protein 1 (PfMSP1) (red) and (D) PfAMA1 (red), respectively, in E64-treated schizonts cultivated with and without GlcN. PfMSP1 was visible in schizonts but forms relatively larger rings that surround multiple nuclei in the agglomerates. PfAMA1 showed loss of signal in agglomerates. Scale bar = 5 μ m. (E) 3D reconstruction of the PfPhIP-HA-glms schizonts with and without GlcN is presented using Imaris 7.6.1. Arrowheads indicate agglomerates showing loss of signal for AMA1 and MSP1 in unsegmented daughter nuclei. See also Figure S5.

Figure 5

PfGAPM2 and PfPhIP function is essential for invasion of the host cell by the merozoites: Merozoites released from GlcN treated (A) PfPhIP-HA-glmS and (B) PfGAPM2-HA-glmS parasites were stained using antibodies against PfGAP50 (green, top panel), PfEBA175 (green, middle panel), PfRON2 (green) and PfAMA1 (red) (lower panel). Staining for these markers showed normal micronemal and rhoptry organelles, however, it was observed that the discharge of contents from these apical organelles was affected. Also, the apical end of the merozoites, attached to the host was not aligned towards the erythrocyte membrane. Scale bar = 5 μ m. (C) 3D reconstruction of the merozoites from PfPhIP or PfGAPM2 deficient parasites arrested on the RBC surface using Imaris 9. See also Figure S6.

Figure 6

Function of PhIL1 associated complex is crucial for reorientation mediated by gliding motor complex and thus host-cell invasion by *Plasmodium falciparum* merozoites: The schematic illustrates GAPM2 and PhIP are essential for blood stage infection and their genetic attenuation arrests merozoite invasion by impeding the function of glideosomal motor machinery resulting in failure of merozoite to reorient its apical end towards the host RBC. Depletion of PhIP also leads to formation of agglomerates of unsegmented schizonts suggesting its probable role in IMC mediated parasite cell division. In the left panel, the level of PfPhIP or GAPM2 protein is maintained by the absence of GlcN. Middle panel shows consequences of PhIP deficiency, wherein a portion of daughter cells get segmented while others remain trapped as an agglomerate under a common plasma membrane. In the agglomerate, IMC fails to form, parasite plasma membrane surrounds multinucleated unsegmented daughter cell, and microneme secretion is affected. Few segmented merozoites which egressed, are seen arrested at the erythrocyte surface and are unable to invade. Right panel summarises the effect of GAPM2 deficiency in the parasite where the merozoites fail to invade the erythrocytes due to the disability of these merozoites to align their apical end towards the host cell surface. Apical reorientation of merozoites is imperative for invasion so that the apical organelles are aligned to the erythrocyte membrane which leads to discharge of apical organelles followed by invasion. Created with BioRender.com

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