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A conserved malaria parasite protein required for maintenance of sporozoite cell shape and transmission

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Malaria parasites are transmitted by mosquitoes and a substantial part of the parasite's complex life cycle takes place inside the insect. Parasite transmission starts with the uptake of parasite stages called gametocytes from the vertebrate host with the blood meal of a female vector mosquito, completing several weeks later with the injection of parasite stages called sporozoites into the vertebrate host by mosquito bite. The sporozoites form in their thousands inside ookinete-derived oocysts situated on the abluminal side of the mosquito midgut epithelium by a process of cell division known as sporogony. After their formation, sporozoites egress from the oocyst into the hemolymph, invade the salivary glands, and mature to become infective to the vertebrate. This MicroCommentary reviews recent reports describing a conserved plasma membrane-associated protein of *Plasmodium berghei*, PBANKA_1422900, and its role in maintaining the shape and structural integrity of sporozoites in salivary glands and during inoculation into the vertebrate host. Combined results from three separate studies provide mechanistic insights into how this protein achieves structural maintenance of the sporozoite, and how in turn this promotes the sporozoite's ability to overcome several physical obstacles and allow it to establish infection in the vertebrate.

Plasmodium sporozoites are responsible for malaria transmission from the insect to the vertebrate host, and to do so must invade the salivary glands of the insect, pass through the salivary ducts, cross the skin and blood vessels to reach and ultimately infect liver cells (Amino *et al.*, 2006, Hopp *et al.*, 2021) (Fig. 1). To achieve this, sporozoites have a slender concave-convex shape and possess specialized secretory organelles (i.e. micronemes and rhoptries) and a unique cortical structure called the pellicle that facilitate invasion and motility (Gubbels & Duraisingh, 2012). The pellicle is defined by a double membrane layer named the inner membrane complex (IMC) that is located directly underneath the plasma membrane (Ferreira *et al.*, 2020). A viscoelastic, subpellicular network (SPN) of intermediate filaments is located at the cytoplasmic side of the IMC (Mann & Beckers, 2001). The SPN is tightly associated with the IMC and forms an internal cytoskeletal basket that supports the IMC membranes and provides tensile strength and flexibility to the cell (Mann & Beckers, 2001, Khater *et al.*, 2004, Tremp *et al.*, 2008, Tremp & Dessens, 2011). The SPN and IMC separate the main cytosol from

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a smaller cortical cytoplasm that contains the molecular machinery that drives sporozoite motility. The latter relies on an actinomyosin motor system that is situated in the space between the plasma membrane and the IMC. The molecular motor and its auxiliary proteins are linked to cell surface adhesins via actin filaments and bridging proteins, and are internally anchored into the IMC (Frenal *et al.*, 2010). Invasion of the salivary glands and infection of the vertebrate host require distinct sporozoite protein repertoires, the expression of which is temporally regulated by programmes of translational repression (Lindner *et al.*, 2019, Bogale *et al.*, 2021). However, many apicomplexan zoite proteins associated with motility and vertebrate infection remain to be characterized (Harding & Frischknecht, 2020). In this MicroCommentary, we review three related studies on one sporozoite protein in the malaria parasite *Plasmodium berghei*, PBANKA_1422900, that has a critical role in maintaining sporozoite shape, integrity and infectivity.

In a study by Singh and colleagues reported in this issue (Singh *et al.*, 2022), the protein PBANKA_1422900 is named Structural Integrity Maintenance Protein (SIMP). The authors demonstrate by gene disruption that SIMP is dispensable for asexual, sexual, ookinete, oocyst and sporozoite stage development. However, SIMP-depleted sporozoites, albeit able to colonise the salivary glands in numbers comparable to wildtype parasites, are not infective to the mouse and unable to establish blood stage infections after intravenous injection of sporozoites. Singh et al (Singh *et al.*, 2022) go on to investigate why SIMP knockout sporozoites are non-infectious. By using circumsporozoite protein (CSP) immunostaining, they show that salivary gland-resident SIMP-depleted sporozoites have an abnormal shape that is characterised by the presence of multiple plasma membrane 'blebs', and further show that these sporozoites display highly reduced gliding motility on glass slides, which is a likely cause of their infectivity loss. These shape and motility phenotypes were reversed in parasites where SIMP function is restored by complementation (Singh *et al.*, 2022). Further cellular investigation of SIMP-depleted sporozoites indicate that they possess an intact cytoskeleton (using phalloidin/actin staining), as well as an intact IMC organisation using immunostaining of the IMC marker PIC5. PIC5 immunostaining also revealed that the IMC does not underlie the plasma membrane blebs indicating that SIMP depletion leads to loss of contact between the plasma membrane and IMC (Fig. 2). This conclusion is indeed supported by their transmission electron microscopic examination of SIMP-depleted salivary gland sporozoites (Singh *et al.*, 2022).

The authors further investigate the localisation of SIMP by generating a transgenic line expressing SIMP fused at its carboxy terminus to a triple hemagglutinin (HA) tag, which allowed them to show by immunostaining that the protein is expressed in gametocytes, ookinetes, sporozoites and liver stages. They show that, in ookinetes and sporozoites, SIMP is predominantly located at the cell periphery, consistent with their *in silico* predictions that SIMP, whilst not possessing any clear known functional domains or motifs, has a potential central hydrophobic membrane-associated domain. Moreover, sporozoites expressing HA-tagged SIMP immunostained under both non-permeabilized and permeabilized conditions, in sharp contrast to PIC5 that only immunostained under permeabilized conditions. The latter observations strongly indicate that the carboxy terminus of SIMP is in fact extracellular. This notion was corroborated by showing that immunostaining with antibodies raised against the carboxy-terminal part of SIMP also does not require sporozoite permeabilization (Singh *et al.*, 2022).

We obtain further insight into the role of PBANKA_1422900 from a second study in which the protein is named 'concavin' (Kehrer *et al.*, 2022). Kehrer and colleagues use carboxy-terminal GFP tagging of the protein combined with immunofluorescence to show its expression in gametocytes, ookinetes, sporozoites and liver stages. The localisation of concavin in ookinetes and sporozoites is shown to be predominantly peripheral, however, immunostaining with anti-GFP antibodies only worked on permeabilized cells leading the authors to conclude that the protein remains intracellular. Further analyses show that the protein is not associated with the SPN protein PHIL1, but co-localises with CSP, collectively pointing to an association with the cytosolic side of the plasma membrane. Kehrer et al. go on to demonstrate that concavin-depleted salivary gland sporozoites over time obtain abnormal shape and display reduced motility (Kehrer *et al.*, 2022). Via electron microscopy and array tomography the authors further show dissociation between the IMC and plasma membrane in concavin-depleted sporozoites.

Concavin-depleted sporozoites are still able to infect mice after mosquito bite, albeit with lower efficacy and increased prepatent periods (Kehrer *et al.*, 2022). indicating that sporozoite infectivity to mice is reduced, but not abolished. The authors go on to show that the reduction in infectivity is not caused by a failure to infect hepatocytes, but rather by reduced efficacy of the sporozoites getting through the narrow salivary ducts, combined with a reduced efficacy to traverse the skin, suggesting that the deformed concavin-depleted sporozoites are having trouble entering and moving through confined spaces (Kehrer *et al.*, 2022). Moreover, they show that a proportion of these sporozoites 'disintegrate' while migrating through the skin. Based on these collective observations, the authors postulate that concavin rivets the plasma membrane to the IMC thereby maintaining the shape of the sporozoite (Kehrer *et al.*, 2022).

A third study of PBANKA_1422900, here named PIMMS22 (Ukegbu *et al.*, 2021), is broadly consistent, demonstrating similar expression and subcellular localisation profiles of the protein as well as a loss of sporozoite infectivity to the mouse in PIMMS22-depleted parasite lines. However, the sporozoite-associated phenotype of PIMMS22-depleted parasites was not investigated in any depth, the study instead focused on an ookinete-associated loss-of-function phenotype (Ukegbu *et al.*, 2021). The authors show that PIMMS22-depleted ookinetes display reduced infectivity as evidenced by significant (4- to 17-fold) reductions in the number of oocysts developing compared to their wildtype counterparts. Ukegbu and colleagues go on to show that PIMMS22 knockout ookinetes have normal motility, ruling out that impaired locomotion is the cause of their infectivity loss (Ukegbu *et al.*, 2021). Instead, the authors conclude that PIMMS22-depleted ookinetes are impaired in midgut invasion as well as ookinete-to-oocyst transition. The findings of this study (Ukegbu *et al.*, 2021) differ from the other two studies where no marked effect on ookinete infectivity was observed (Kehrer *et al.*, 2022, Singh *et al.*, 2022). As distinct mosquito species were used in assessing parasite development, this discrepancy is explained by differences in susceptibility of *Anopheles* species to *Plasmodium* infection (Singh *et al.*, 2022). Indeed, several ookinete surface molecules have been reported as having roles in evading the insect's innate complement-like immune responses to parasite infection (Molina-Cruz *et al.*, 2013, Ukegbu *et al.*, 2020, Ukegbu *et al.*, 2017) and this result raises the prospect that PIMMS22, too, could play a role in this aside from its role in sporozoite infectivity.

The importance of sporozoite shape for infectivity to the mouse has been demonstrated with other pellicle proteins that are required for normal sporozoite shape and motility, for example the SPN-resident alveolin IMC1h (Tremp & Dessens, 2011, Volkmann *et al.*, 2012, Coghlan *et al.*, 2019). IMC1h-depleted sporozoites have reduced motility and cannot infect mice via natural mosquito bite or via subcutaneous needle injection, but they can establish infections after intravenous needle injection (Volkmann *et al.*, 2012). Moreover, IMC1h null mutant sporozoites display normal hepatocyte infection rates *in vitro* (Volkmann *et al.*, 2012), as is indeed the case for concavin null mutants (Kehrer *et al.*, 2022). The identified role of PBANKA_1422900 in maintaining sporozoite shape and integrity further supports the notion that traversal of the dermis constitutes a major bottleneck for sporozoite infection of the vertebrate host.

What remains somewhat puzzling is why depletion of protein PBANKA_1422900 has no discernible effect on ookinete shape or motility, even though the protein appears to have the same subcellular localisation as it does in sporozoites. One explanation is that the protein has an entirely different function in ookinetes, something that the Ukegbu et al study (Ukegbu *et al.*, 2021) might be alluding to. It is also possible that the life span of ookinetes simply is not long enough for their cell shape to be impacted by the absence of the protein. Contrary to sporozoites, which constitute the final life stage in the insect and live for many days, ookinetes quickly transform into oocysts after crossing the mosquito midgut epithelium. It would be interesting to investigate if there is a longer-term effect on the shape or integrity of cultured ookinetes depleted of PBANKA_1422900.

There is still discrepancy whether PBANKA_1422900 has an extracellular part or not. Whilst the studies by Singh et al (Singh *et al.*, 2022) and by Ukegbu et al (Ukegbu *et al.*, 2021) support an extracellular localisation of at least part of the protein, the Kehrer et al study (Kehrer *et al.*, 2022) does not. This is relevant in the context of several malaria intervention strategies. First, malaria vaccine based on immunodominant sporozoite antigen like CSP (Kumar *et al.*, 2006) target the sporozoite's ability to establish parasite infection after mosquito bite (Cowman *et al.*, 2016). The relatively abundant PBANKA_1422900, if indeed extracellular, could be a valuable addition to the list of urgently needed new malaria vaccine candidates (Matuschewski, 2017). In addition, given its potential surface expression on ookinetes, transmission-blocking vaccines could be generated based on PBANKA_1422900. This type of vaccine target gametocyte and/or ookinete surface proteins via antibodies taken up by the mosquito with its *Plasmodium*-infected blood meal to block parasite development in the midgut (Delves *et al.*, 2018). Thus, further scrutiny of the potential extracellular nature of this protein is warranted.

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Figure legends

Fig. 1 Malaria parasite life cycle.

Fig. 2 Cartoon of *Plasmodium berghei* wildtype (WT) and SIMP-depleted (SIMP KO) salivary gland sporozoites depicting the plasma membrane (PM) and inner membrane complex (IMC). Detachment of the PM and IMC in SIMP KO sporozoites leads to an abnormal sporozoite shape and rounding of the cell.

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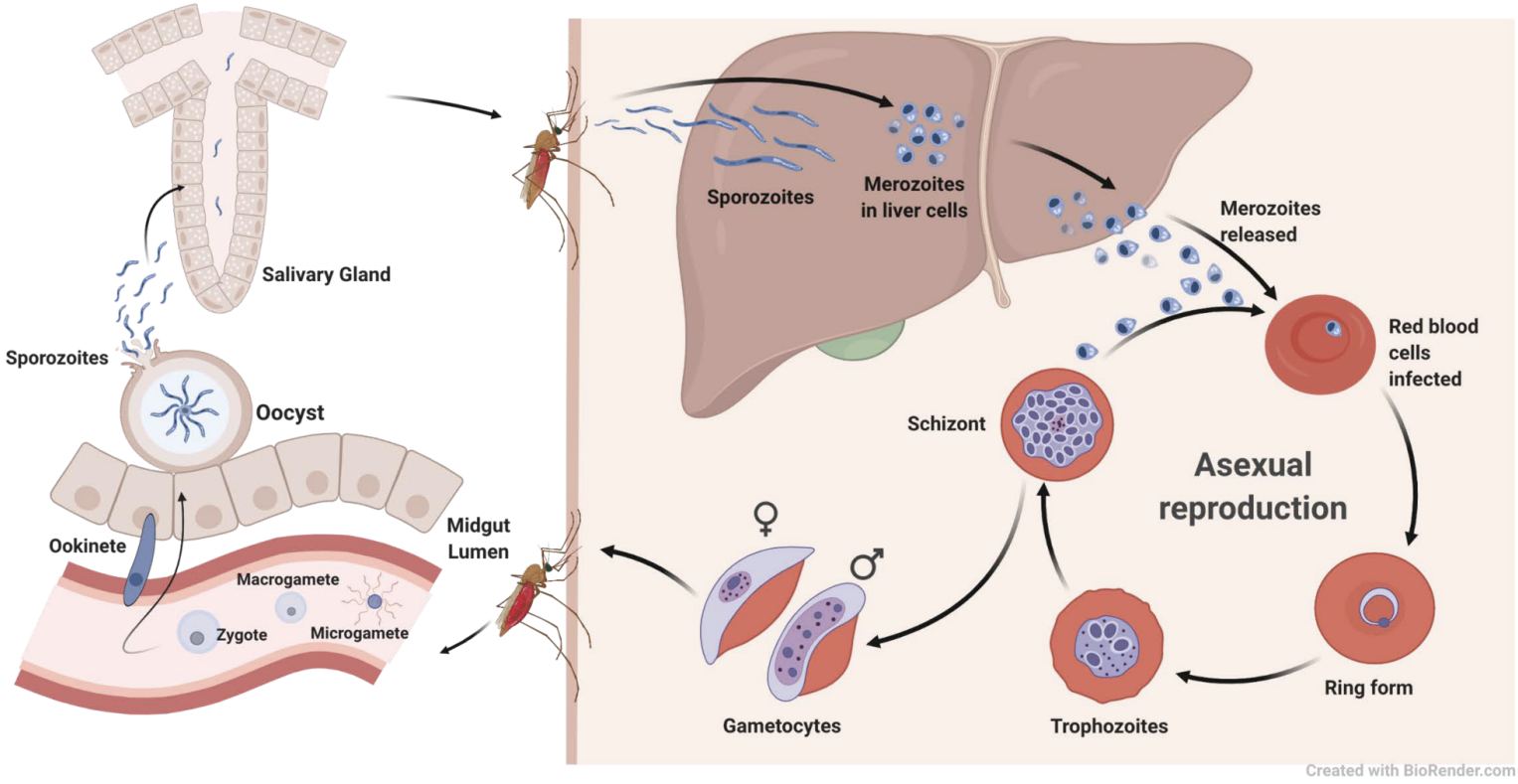
The *Plasmodium berghei* protein PBANKA_1422900 is associated with the plasma membrane (PM) of ookinetes and sporozoites. Depletion of PBANKA_1422900 causes detachment of the PM and inner membrane complex, resulting in a gradual loss of sporozoite shape, structural integrity and infectivity.



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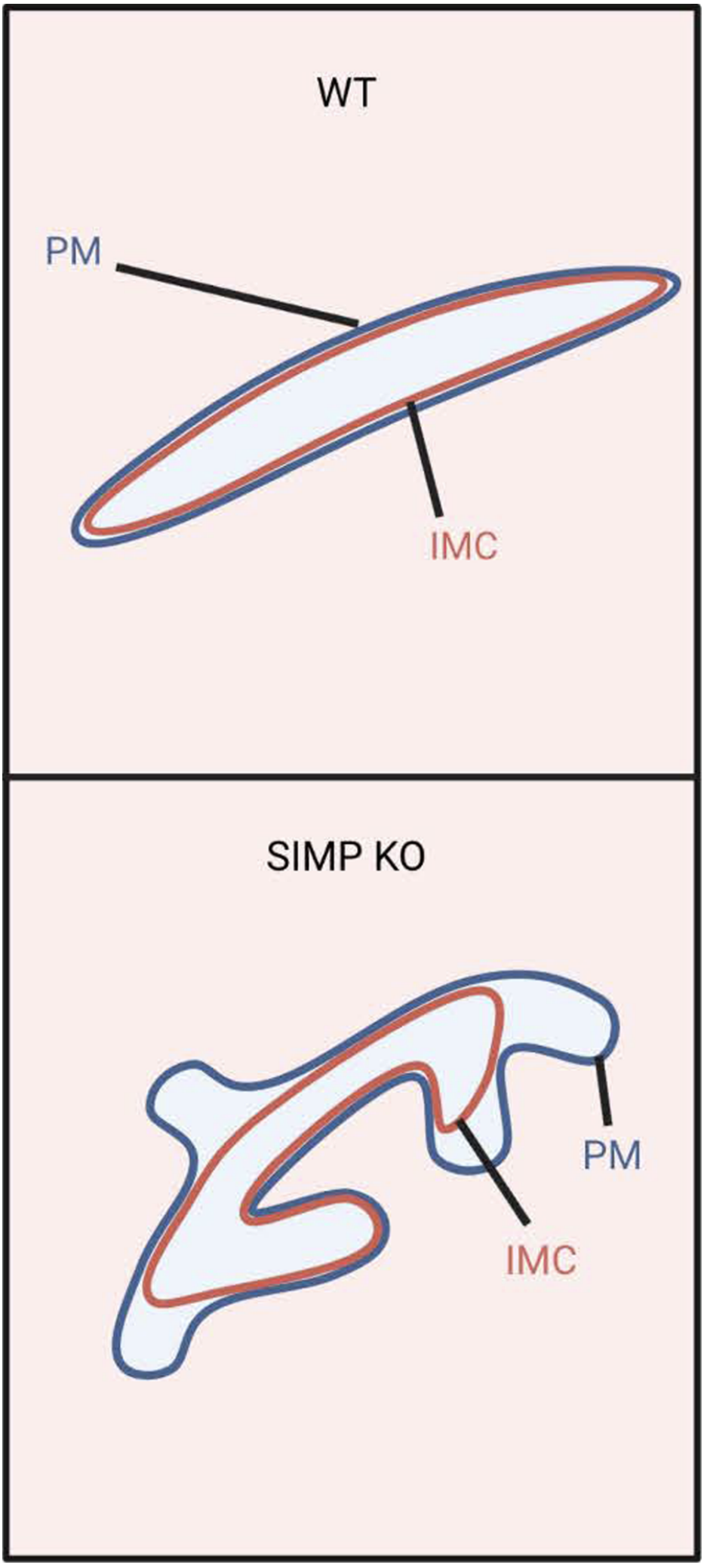

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