SPOROZOITE METAMORPHOSIS IN THE LIVER: INVESTIGATION OF THE ROLE OF

*Plasmodium* GRASP AND VPS4 IN MICRONEME ELIMINATION

by

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Abstract

The *Plasmodium* metamorphosis during liver stage requires morphologic and metabolic changes including clearance of micronemes, previously required for hepatocyte penetration. Experimental evidence indicates that *Plasmodium* utilizes machinery associated with unconventional protein secretion (UPS) pathway for micronemal clearance from liver stage. In mammalian cells, the UPS-related pathway is involved in secreting proteins in double-membrane vesicles from endoplasmic reticulum to plasma membrane, bypassing the Golgi, instead requiring autophagy related genes 8 (ATG8), Golgi reassembly and stacking protein (GRASP) and vacuolar protein sorting-associated protein 4 (VPS4), in a process called exophagy. In exophagy, GRASP has been identified to localize autophagosomes or multivesicular bodies (MVB) and mediate their tethering to the plasma membrane for cargo expulsion. Previous investigations reveal colocalization of PbGRASP with PbATG8 and PbVPS4, proteins essential for microneme compartmentalization and MVB biogenesis (hypothesized), respectively. To investigate the role of PbGRASP during liver stage development, the laboratory engineered a *Plasmodium* mutant, named PbGRASP-KO (*Plasmodium berghei* GRASP knockout). Analysis of the parasite size between the PbGRASP-KO and WT, as the sporozoite develops into its exoerythrocytic forms (EEF), suggest that PbGRASP may be involved in early-mid stage schizogony however is dispensable for liver stage development as seen through successful schizogony. At 22h post infection, the PbGRASP-KO EEF forms small schizonts. From quantification of TRAP signal in IFA and its normalization to EEF volume, we observe that PbGRASP is non-essential for microneme elimination. To understand the association of GRASP with exophagy machinery, we attempted to develop a PbGRASP-specific antibody in mice through intraperitoneal immunization. The antibody was generated against a synthetic multi-epitope protein of PbGRASP, designed, generated, and purified in laboratory. Western blot and immunofluorescence analysis of the antibody activity compared between WT and PbGRASP-KO suggests that these antibodies detect non-specific proteins rather than PbGRASP as seen from lack of
clear and distinct puncta of PbGRASP in WT. In addition, we are in the process to clone a new Plasmodium mutant, named PbVPS4-KO (Plasmodium berghei VPS4 knockout) to investigate the role of VPS4 in Plasmodium development.

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Secondary Reader: Dr. Photini Sinnis, MD
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<tbody>
<tr>
<td>αMEM</td>
<td>αMinimum Essential Medium</td>
</tr>
<tr>
<td>ACP</td>
<td>Acyl carrier protein</td>
</tr>
<tr>
<td>ACT</td>
<td>Artemisinin-based combination therapies</td>
</tr>
<tr>
<td>ATG</td>
<td>Autophagy-related genes</td>
</tr>
<tr>
<td>bp</td>
<td>Base Pairs</td>
</tr>
<tr>
<td>CDC</td>
<td>Center for Disease Control</td>
</tr>
<tr>
<td>CPS</td>
<td>Conventional Protein Secretion</td>
</tr>
<tr>
<td>CSP</td>
<td>Circumsporozoite protein</td>
</tr>
<tr>
<td>CUPS</td>
<td>Compartments for unconventional protein secretion</td>
</tr>
<tr>
<td>DAPI</td>
<td>4′,6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>EEF</td>
<td>exo-erythrocytic form</td>
</tr>
<tr>
<td>EM</td>
<td>Electron microscopy</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic Reticulum</td>
</tr>
<tr>
<td>ERES</td>
<td>Endoplasmic Reticulum exit sites</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>FPLC</td>
<td>Fast Protein Liquid Chromatography</td>
</tr>
<tr>
<td>gDNA</td>
<td>Genomic deoxyribonucleic acid</td>
</tr>
<tr>
<td>GRASP</td>
<td>Golgi Reassembly and Stacking Protein</td>
</tr>
<tr>
<td>h</td>
<td>hours</td>
</tr>
<tr>
<td>hDHFR</td>
<td>Human Dihydrofolate Reductase</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish Peroxidase</td>
</tr>
<tr>
<td>HSPGs</td>
<td>Heparin Sulphate Proteoglycans</td>
</tr>
<tr>
<td>IFA</td>
<td>Immunofluorescence assay</td>
</tr>
<tr>
<td>IMC</td>
<td>Inner membrane complex</td>
</tr>
<tr>
<td>iRBCs</td>
<td>infected Red Blood Cells</td>
</tr>
<tr>
<td>IRS</td>
<td>Indoor Residual Spraying</td>
</tr>
<tr>
<td>LB</td>
<td>Luria-Bertani</td>
</tr>
<tr>
<td>LLINs</td>
<td>Long lasting insecticide treated bed nets</td>
</tr>
<tr>
<td>LS</td>
<td>Liver stage</td>
</tr>
<tr>
<td>MAC/PF</td>
<td>Membrane Attack Complex/perforin-like domain</td>
</tr>
</tbody>
</table>
min minutes
MJ Moving Junction
mRNA Messenger ribonucleic acid
MSP1 Merozoite surface protein 1
MVB Multivesicular bodies
p.i. post infection
Pb Plasmodium berghei
PbATG8-OE Plasmodium berghei Autophagy-related gene 8 overexpressor mutant
PBS Phosphate-buffered saline
PCR Polymerase chain reaction
Pf P. falciparum
PLP1 Perforin-like protein 1
PS Penicillin-Streptomycin
PV Parasitophorous Vacuole
PVM Parasitophorous Vacuolar Membrane
RBC Red Blood cell
RDTs Rapid Diagnostic Tests
RT room temperature
sec seconds
SERCaP Single Encounter Radical Cure and Prophylaxis
SPECT2 Sporozoite microneme protein essential for traversal 2
synGRASP synthetic GRASP protein
synGRASP-U synthetic GRASP protein eluted in buffer containing urea
synGRASP-P synthetic GRASP protein in PBS buffer
TLP TRAP-like protein
TRAP Thrombospondin-related anonymous protein
UPS Unconventional Protein Secretion
UTR Untranslated region
VPS4 Vacuolar protein sorting-associated protein 4
WT Wild type
Introduction

I. Epidemiology and Current Interventions

Malaria is a globally prevalent, deadly parasitic disease caused by Plasmodium spp., and transmitted through the bite of an infected mosquito. The five main Plasmodium species responsible for infecting humans are P. falciparum, P. vivax, P. malariae, P. ovale and P. knowlesi, which differ in the severity of infection and the geographic prevalence (Fletcher & Beeching, 2013). However, endemicity of Plasmodium spp. to 84 tropical and sub-tropical countries spanning Africa, Asia and Americas puts more than half of the population at risk (World Health Organization, 2022). The high burden of malaria in the endemic regions also contributes to their poor socio-economic development making malaria a major concern for public health (Gallup & Sachs, 2001).

In 2021, around 247 million cases were reported in endemic regions with the number of deaths amounting to 619,000 (World Health Organization, 2022). Although implementation of elimination and prevention programs has helped avert an estimated 2 billion cases and 11.7 million deaths globally in the period 2000-2021, disruptions during COVID-19 pandemic have led to an additional preventable 13.4 million cases and 63,000 deaths (World Health Organization, 2022). In endemic countries, the burden falls largely on population groups of infants, children under 5 years of age, pregnant women, people with HIV and travelers with low/compromised immunity due to a higher risk of contracting infection and developing severe malaria (Fletcher & Beeching, 2013). In 2021, 32% of pregnant women in Africa were still exposed to infection with children under 5 years of age accounting for 76% of global malaria deaths, which has stalled since 2015, suggesting the importance of population-group specific interventions in controlling the burden of malaria (World Health Organization, 2022).
The current interventions to reduce incidence of malaria are based on vector control, preventive chemotherapies and vaccines. To control vector populations and vector-human interactions in endemic regions, households are recommended to use long lasting insecticide treated bed nets (LLINs) and indoor residual spraying (IRS) with insecticides. However, emergence of resistance against at least two insecticides including pyrethroid, predominantly used in IRS and LLINs (Toé et al., 2014), and behavioral adaptations in mosquitoes to early feeding, feeding and resting outdoors (Gatton et al., 2013), allow for residual transmission. Therefore, development of newer approaches including mosquito treatment with ivermectin (Chaccour et al., 2013), sugar baits and spatial repellents are required to counter the residual transmission (Killeen et al., 2017).

Along with vector control, mass drug administration in endemic regions for chemoprophylaxis, especially for high-risk populations, is implemented regularly. For instance, pregnant-women and children are subjected to intermittent preventive therapy with different anti-malarial regimens (Desai et al., 2015). In Sahel, where malaria is highly seasonal, children are treated at monthly intervals during the transmission season as part of Seasonal Malaria Chemoprevention resulting in a reduction of 80% in incidence and 60% in deaths (Cissé et al., 2016). Additionally, travelers to these countries are put on fixed-term prophylaxis with a combination of drugs, based on the travel destination, following recommendations of the WHO or the US Center for Disease Control (CDC). Although, most of the drugs used are non-artemisinin drugs such as sulfadoxine and pyrimethamine, emergence of resistance against existing drugs remains to be a constant fear (Desai et al., 2015).

The development of an effective vaccine for malaria has been a challenging process due to the large genome of *Plasmodium* spp. (Gardner et al., 2002) and its high antigenic variability (de la Cruz et al., 1987; Mkumbaye et al., 2017). Various strategies have been utilized to design vaccines for malaria targeting different stages of the *Plasmodium* lifecycle (Zheng et al., 2019). Although, these vaccine candidates have largely proven to be ineffective in naïve people, for the very few vaccine candidates that have been successful and effective against non-exposed individuals in clinical trials, usage of same
regimen in endemically exposed individuals resulted in reduced efficacy (Jongo et al., 2018; Olotu et al., 2018) due to the tolerance created by the naturally acquired immunity (Kumar et al., 2019; Langhorne et al., 2008). Finally, the RTS,S/AS01 vaccine targeting the circumsporozoite protein (CSP) (Gordon et al., 1995), expressed on the surface of the sporozoite, is the only vaccine recommended for use by World Health Organization in 2021 (World Health Organization, 2021). RTS,S/AS01 is a subunit vaccine derived from the genetic fusion of CSP and surface protein, S, of Hepatitis B virus and supplemented with the adjuvant AS01 (Cohen et al., 2010). The chimeric protein is expressed by Saccharomyces cerevisiae yeast cells to assemble as virus-like particles (Cohen et al., 2010). A phase 3 study of RTS,S/AS01 with a 3-dose regimen showed an efficacy of 55.1% and 47.3% in preventing clinical malaria and severe malaria, respectively, in children between the ages of 5 and 17 months over 12 months after the third dose (Syed, 2022). Although the immunity against clinical malaria is short lived with efficacy falling to 26% against clinical malaria with no efficacy against severe malaria, after 4 years of vaccination in children, a booster dose increases the overall efficacy to 39% and 28.5%, respectively (Syed, 2022), reducing cases of severe malaria in children by 30% (World Health Organization, 2021). Hence, WHO recommends its use among children living in regions of moderate to high malaria transmission in a 4-dose schedule (World Health Organization, 2022).

II. Case Management

Malaria initially presents itself with fever, malaise, fatigue, chills, body-aches, headache and abdominal discomfort, among others. If left untreated, the uncomplicated malaria progresses into severe malaria leading to cerebral malaria, acute respiratory distress syndrome, acute kidney injury or acidosis causing shock and multi-organ failure. In children, it is also accompanied by severe malarial anemia (Moraleda et al., 2017).
Current diagnostic tools for uncomplicated malaria are designed to confirm the presence of the parasite since the signs and symptoms are non-specific and hard to distinguish from flu-like syndromes including COVID-19 (Konozy et al., 2022) Light microscopy of highly sensitive thick blood films helps detect presence of infection in red blood cells and thin films can be used to differentiate between Plasmodium species and quantify the parasitemia (World Health Organization, 2018). However, rapid diagnostic tests (RDTs) are predominantly used as first line diagnostics, as they are widely available, affordable, cost-effective and require minimal training. Blood collected from a finger prick is used to test for PfHRP-2 gene for *P. falciparum* (Pf) infections, lactate dehydrogenase enzyme for pan-malarial or species-specific infections, or aldolase antigens, providing simple, sensitive and specific diagnosis but with limitations (World Health Organization, 2018). RDTs can fail to work giving false positives weeks post parasite clearance or false negatives during infections with genetically evolved *P. falciparum* lacking PfHRP-2/3 genes or during hyperparasitemia (Gillet et al., 2009).

Artemisinin- and quinolone-based antimalarials are used to treat people diagnosed with malaria. Over time, artemisinin derivatives have taken precedence over quinolone-based drugs as they are highly effective, fast-acting, safe, potent and well tolerated. Although chloroquine is still a WHO recommended drug for treating uncomplicated malaria of *P. vivax*, wide-spread emergence of resistance against chloroquine and antifolates in *P. falciparum* and in few regions of *P. vivax*, has led to the usage of artemisinin-based combination therapies (ACT) to counter the multi-drug resistant parasites (Amelo & Makonnen, 2021). ACTs use a combination of artemisinin-derivatives to rapidly reduce parasitemia and a partner drug to clear residual parasitemia. However, insufficient supply of quality-assured and affordable ACT (World Health Organization, 2017) has led to the emergence of partial resistance against artemisinin resulting in a slower parasite clearance (Menard & Dondorp, 2017). Hence, the proposed solution to reduce risk of resistance and transmission is Single Encounter Radical Cure and Prophylaxis (SERCaP), a drug active at multiple stages of *Plasmodium* lifecycle. A single dose of SERCaP provides long-lasting prophylactic treatment and eliminates all the malaria parasites in the body effectively blocking
transmission and preventing relapse (The malERA Consultative Group on Drugs, 2011). The target drug profile of SERCaP if developed would be very beneficial but achieving an operative drug regime with age-appropriate dosage and an affordable price is difficult to achieve (White & Nosten, 2021).

III. Biology and Lifecycle

The unicellular protozoan *Plasmodium* sp., causative agent of malaria, belongs to the Apicomplexa phylum of obligate intracellular parasites, which invade host cells in specialized form called zoites. The structurally conserved zoites are elongated, polarized cells that glide on solid surfaces without changing their shape, secrete proteins from secretory organelles called micronemes and rhoptries from their apical end, and form a specialized membrane-bound compartment, parasitophorous vacuole (PV), when invading host cells to further develop and replicate (Tardieux & Ménard, 2008). The lifecycle of *Plasmodium* is conserved across species and can be divided between its two organisms – a vertebrate host as intermediate host and an insect vector as definitive host, which is *Anopheles* spp. mosquitoes for mammalian infecting *Plasmodium* spp. (Figure 1).

When a female *Anopheles* mosquito feeds on a host for a blood meal, it injects saliva while probing the skin to reach the bloodstream. During the probing phase of an infectious bite, a small number of sporozoites are released in its saliva and inoculated in the host dermis (Frischknecht & Matuschewski, 2017). Once, the mosquito is fed, the remaining sporozoites in the saliva are reingested into the mosquito midgut (Frischknecht & Matuschewski, 2017). The *Plasmodium* sporozoites are 8-14 µm long, crescent shaped zoites that use either substrate-dependent active motility known as gliding motility for transport or use passive transport in the host’s fluids (Frischknecht & Matuschewski, 2017; Tardieux & Ménard, 2008). This pointed and elongated shape and motility of the sporozoite is maintained until hepatocyte infection by a cytoskeleton composed of microtubules and an inner membrane complex (IMC), an organelle forming a double membrane layer beneath the parasite’s plasma membrane (Tardieux &
The inoculated sporozoites can take three possible pathways to exit the dermis: (i) invade blood capillaries to reach liver and infect hepatocytes, (ii) invade lymphatic vessels and get degraded by dendritic cells, or (iii) remain in the dermis after the cessation of active motility (Tardieux & Ménard, 2008).

In 1-3 hours (h) of inoculation, a portion of the sporozoites glide to reach and enter the bloodstream using the TRAP-like protein (TLP), necessary for escaping the dermis (Moreira et al., 2008). Through the circulation system, the sporozoites access the liver by crossing the sinusoidal barrier of epithelial cells and macrophage-like Kupffer cells, a process also known as cell traversal (Tavares et al., 2013). During cell traversal, to pass through cells, the sporozoites can either breach the cell plasma membrane (Mota et al., 2001) or form transient vacuoles that are ruptured before exit and egress using pH sensing and SPECT2 (sporozoite microneme protein essential for traversal 2 or perforin-like protein 1, PLP1), a protein with a membrane attack complex/perforin-like domain (MAC/PF) that forms pores on the cell membrane (Risco-Castillo et al., 2015). Although the primary purpose of cell traversal is to cross the liver sinusoids (Tavares et al., 2013) and ensure sporozoite survival during the process by defending against host phagocytic leukocytes (Tardieux & Ménard, 2008), it also primes them to infect hepatocytes.

After traversing several hepatocytes, the sporozoites switch from ‘migratory mode’, that starts in dermis, to ‘invasive mode’ at hepatocyte (Dundas et al., 2019; Tavares et al., 2013)(Dundas et al., 2019; Tavares et al., 2013). This switch is signaled by the binding of CSP, coating the sporozoite, to the higher sulphated forms of heparin sulphate proteoglycans (HSPGs) of the hepatocytes (Coppi et al., 2007; Herrera et al., 2015). In addition, the micronemal protein thrombospondin-related anonymous protein (TRAP) with adhesive domains also plays an important role in hepatocyte invasion (Paoletta & Wilkowsky, 2022; Sinnis & Sim, 1997). At the site of close apposition of host and parasite plasma membranes, moving junction (MJ) is assembled by sporozoites to invade host cells (Loubens et al., 2021). On successful infection, MJ resolves as a ring through which the parasite penetrates inside an invagination of host plasma membrane, initiating the formation of PV membrane (PVM) (Loubens et al.,
The PVM is remodeled during invasion to selectively exclude some host membrane proteins, resulting in a nonfusogenic PVM that prevents fusion with host lysosomes and any other organelles (Loubens et al., 2021). Post invasion, the PVM is further remodeled to include parasite-derived proteins important for liver-stage development including UIS4 (Loubens et al., 2021). Inside the hepatocyte, the permeability of the PVM can be highly regulated by the parasite to access cytosolic nutrients through channels within the PVM (Spielmann et al., 2012) and small pores that restrict the passage of solutes to less than 855Da (Bano et al., 2007).

Once hepatocyte invasion is successful, sporozoite initiates schizogony, dedifferentiating into the liver stage (LS) or exo-erythrocytic form (EEF) over the next 2-10 days according to the species (Bano et al., 2007). As the sporozoite is transforming, it compartmentalizes micronemes and eliminates the TRAP-associated ones from the parasite to give way to the new micronemes containing specific proteins such as AMA-1 for erythrocyte invasion (Jayabalasingham et al., 2010). Therefore, the metamorphosis leads to the loss of rhoptry-microneme complex followed by the loss of IMC and microtubules of the cytoskeleton (Meis et al., 1985). The parasite growth during transformation, also results in dramatic expansion in its volume and generation of new organelles without programming host cell death (Meis et al., 1985; Sturm et al., 2006). At the end of schizogony, about 40,000 merozoites per hepatocyte bud out of the cell in parasite-filled vesicles called merosomes and are released into the bloodstream (Bano et al., 2007).

The blood stage of *Plasmodium* is responsible for clinical manifestation of malaria due to the repeated asexual cycles of invasion, replication, egress and reinvasion initiated by the merozoites. Within 30-90 seconds (sec) of its release, merozoite invades new erythrocytes by reorienting its apical end to form a MJ with erythrocytes and release contents from micronemes and rhoptries (Gilson & Crabb, 2009). Analogous to the liver-stage, the parasite invaginates into the host cell forming a PV containing both host and parasite proteins (Goldberg & Zimmerberg, 2020). Inside the PV, the parasite increases its volume and takes up hemoglobin from the host (Goldberg & Zimmerberg, 2020). While the digested hemoglobin is used for protein synthesis, the undigested heme is deposited as hemozoin (Goldberg &
Zimmerberg, 2020). Inside the erythrocytes, the invasion competent hepatic merozoites first transform to the ring stage, progressing into trophozoites and schizonts. On maturation, the schizonts lyse RBCs producing multiple merozoites for reinvasion, completing the asexual cycle (Gilson & Crabb, 2009).

On reinvasion, a small portion of asexual blood-stage parasites undergo gametocytogenesis to sexually differentiate into male and female gametocytes. When an Anopheline feeds on an infected host, it ingests the transmission-competent gametocytes from the blood, which rapidly undergo maturation to produce sexually competent gametes inside the mosquito (Josling & Llinás, 2015). The male gametes fertilize female gametes to produce a zygote, which develops into an oocinete. The oocinete travels through the midgut epithelial cells, initiating the sporogony (Frischknecht & Matuschewski, 2017; Josling & Llinás, 2015). During sporogony, the oocinete transform into oocysts, within which sporozoites develop (Frischknecht & Matuschewski, 2017). On rupturing, the oocysts produce sporozoites into the hemolymph (Frischknecht & Matuschewski, 2017). Through the hemolymph, the sporozoites migrate to the salivary gland to reside until the mosquito delivers them during the next blood meal, continuing the life cycle (Frischknecht & Matuschewski, 2017).

IV. Autophagy during *Plasmodium* metamorphosis in the liver

*Plasmodium* undergoes dramatic morphologic and metabolic changes as it transforms inside unique tissues of an insect and vertebrate hosts. For instance, during the intracellular development inside the liver, the *Plasmodium* transforms from a motile, long and slender, invasion ready sporozoite to a rounded, metabolically active, replication-competent trophozoite. necessary for the development of sporozoite into a replication-competent trophozoite (Coppens, 2011). To invade hepatocytes, the sporozoite discharges contents from its two secretory organelles, micronemes and rhoptries, at the time of host-cell contact. As noted previously, micronemal protein TRAP contributes to hepatocyte adhesion while content of rhoptries is implicated in the biogenesis of PV (K. J. Robson et al., 1995; K. J. H.
Robson et al., 1988; Sinnis & Sim, 1997; Tufet-Bayona et al., 2009). From 4h post-infection (p.i.) onwards, sporozoite undergoes multiple spectacular phenotypic changes starting from the bulging in the middle of the parasite leading to sphericalization as the two distal ends of sporozoite retract and disappear (Figure 2A, 2B). This structural makeover is accompanied by the clearance of micronemes and rhoptries, superfluous for parasite replication, and the discharge of cytoskeleton and IMC. Before release, micronemes are sequestered in a double membrane-bound compartments that are morphologically similar to autophagosomes. The double membrane-bound compartments accumulate at the parasite periphery and dock on the plasma membrane, suggesting exocytic events through autophagy (Brennand et al., 2011; Coppens, 2011; Duszenko et al., 2011; Jayabalasingham et al., 2010).

Autophagy is a biological process many organisms use to discard organelles during differentiation (Dupont et al., 2011; Glick et al., 2010). It can also be used to eliminate redundant, senescent, damaged proteins or organelles, provide nutrients to the cell, or degrade intracellular pathogens, colloquially referred to as “housekeeping” activities to maintain homeostasis (Dupont et al., 2011; Glick et al., 2010). Eukaryotic cells use conventional protein secretion (CPS) as a “standard” method for trafficking of proteins. In CPS pathway, the cargo proteins at the endoplasmic reticulum (ER), for protein folding and maturation, are recognized through a transmembrane domain signal peptide (leader sequence) and trafficked to the Golgi organelle for further processing and packaged into secretory vesicles that are sent to a final destination (Kim et al., 2018). During cell stress, such as starvation or mechanical stressors, eukaryotic cells use unconventional protein secretion (UPS) for proteins lacking a signal peptide (Kim et al., 2018). The UPS pathway is however not limited to leaderless peptides as proteins with a signal peptide can also undergo unconventional secretion bypassing Golgi complex during cellular stress and development (Gee et al., 2018). Diverse UPS pathways have been identified involving both vesicular and non-vesicular transport, bypassing the classic secretory pathway with vesicular UPS pathway requiring autophagy machinery (Kim et al., 2018). Proteins are engulfed into an autophagosome
that fuse with multivesicular bodies (MVB) to form amphisomes that fuse with the plasma membrane to release the cargo.

*Plasmodium* spp. possess a rudimentary set of autophagy-related gene (ATG) proteins, responsible for the function and regulation of UPS pathway (Brennand et al., 2011; Coppens, 2011; Duszenko et al., 2011; Jayabalasingham et al., 2010; Wesselborg & Stork, 2015). Transcriptomic analysis suggests that autophagy orthologs found in *P. falciparum* have a role in induction and selection of cargo, the formation of a phagophore, assembly of the autophagosome from the phagophore, and degradation of the autophagic body membrane (Cervantes et al., 2014; Navale et al., 2014). Further, transcripts of ATG3, ATG7, and ATG8 from the ATG8-complex involved in the expansion of the phagophore are detected in all *Plasmodium* stages but are upregulated in parasites upon liver infection at day 1 (Jayabalasingham et al., 2014). In *Plasmodium berghei*, *Pb*ATG8 is solely membrane-associated and colocalizes with micronemal TRAP. Double immunostaining reveals that the overlap of micronemal TRAP and *Pb*ATG8 increases with progress of conversion process up to 12h p.i. (Figure 2C), suggesting a role of the parasite autophagic machinery in microneme sequestration. Furthermore, *Pb*ATG8 localizes to structures that align along the apicoplast identifiable with the acyl carrier protein (ACP) marker (Figure 2D). The apicoplast is delineated by four membranes and immunogold staining reveals that *Pb*ATG8 is located on the two outermost membranes of the apicoplast (Figure 2E), which are enriched with phosphatidylinositol 3-phosphate (Tawk et al., 2010), a lipid that marks mammalian autophagic structures (Axe et al., 2008; Dall’Armi et al., 2013). *Pb*ATG8 is also found on membranes of vesicles and tubules close to the apicoplast. This suggests that the apicoplast is the source of membranes for phagophore and autophagosome formation. In *P. falciparum*, *Pf*ATG8 is revealed to be present and expressed across multiple sexual and asexual stages of parasite development. Additionally, abolishment of apicoplast organelle through chloramphenicol treatment resulted in disruption of *Pf*Atg8-GFP branching during division (Roestenberg et al., 2011). Moreover, *Pf*ATG8 knock-down parasites were found to be lacking a functional apicoplast and failed to successfully replicate.
Macroautophagy is associated with the formation of autolysosomes by fusion of autophagosomes with degradative organelles or lysosomes for cargo degradation. As intrahepatic *Plasmodium* lacks lysosomes (Voss et al., 2016), converting parasites employ an alternative UPS-like pathway, secretory autophagy (named exophagy), to eliminate unwanted organelles sequestered into autophagosomal structures, as described in other organisms during differentiation (Giuliani et al., 2011). In exophagy, autophagosomes bypass fusion with lysosomes and instead fuse with the plasma membrane to release their content outside the cell. Exophagy requires the association of the Golgi protein, GRASP with autophagosomes to trigger the fusion of autophagosomes with multivesicular bodies containing VPS4 to form hybrid amphisomes that fuse with the plasma membrane (Hanson & Cashikar, 2012; Vinke et al., 2011). *Plasmodium* contains genes coding for GRASP and VPS4 homologs (Struck et al., 2005; Yang et al., 2004) and GRASP and VPS4 transcripts are expressed in intrahepatic *P. berghei* 24h p.i. (Voss et al., 2016).

Based on these data, a working model has been developed to outline the cellular events that lead to the elimination of micronemes through exophagy during sporozoite development inside hepatocytes (Fig 2F). In this model, the apicoplast supplies PbATG8-containing membranous structures to generate phagophores (step I); micronemes are sequestered into phagophores and autophagosomes (step II); GRASP is transported from the Golgi to autophagosomes for membrane fusion (step III); autophagosomes fuse with MVB to form amphisomes (step IV); amphisomes fuse with the plasma membrane for microneme release into the PV (step V) and microneme are degraded within the PV (step VI). To validate step IV of this model and illustrate an exophagic pathway intersecting with Golgi vesicles and multivesicular bodies, liver forms were immunostained for ATG8, GRASP and VPS4. VPS4-and GRASP-positive autophagic compartments were detected in converting parasites (Figure 2G). This result points to a potential cross-talk between the autophagic machinery and exocytic organelles for organelle disposal in liver forms.
To examine whether that PbATG8 plays a key role in microneme elimination by exophagy in intrahepatic *Plasmodium*, PbATG8 expression has been manipulated by creating a strain that conditionally overexpresses ATG8 (PbATG8-OE parasites (Voss et al., 2016)) when sporozoites reach the salivary glands and thus in the liver. This strain has an insertion of a 60-bp ‘GC’-rich sequence in the 3’ UTR of ATG8, resulting in the stabilization of ATG8 mRNA and a 2-fold increase of ATG8 expression during liver stage compared to the parental strain. Examination of PbATG8-OE infection in hepatocytes reveals that PbATG8-OE parasites suffer developmental defects; delayed karyokinesis and schizogony, abnormally small PV, and undersized merosomes, the membrane package produced at the end of schizogony that contains hepatic merozoites (Sahu et al., 2021; Voss et al., 2016). These developmental defects of PbATG8-OE parasites in the liver lead to delays in initiating the first round of blood infection. Other abnormalities found in these parasites were expanded apicoplasts (the PbATG8 signal looks branched into a reticulate network) and delayed microneme evacuation into the PV (Voss et al., 2016). Together, these data confirm the participation of *Plasmodium* autophagy and ATG8 in the development of the parasite infection in hepatocytes.

V. Secretory autophagy in sporozoites: role of *Plasmodium* GRASP and VPS4 for microneme elimination

Multicellular eukaryote organisms possess hundreds of stacked Golgi organelles per cell that are either interconnected to form a continuous ribbon structure such as in vertebrates, or dispersed throughout the cytoplasm such as in plants (Dupree & Sherrier, 1998; Wei & Seemann, 2010). Although the secretory function of the Golgi apparatus is highly conserved throughout the eukaryotic kingdom, unicellular eukaryotes contain Golgi cisternae that are either piled up to form only one single stack (e.g., in trypanosomes, *Toxoplasma*) or that are not assembled but spread in the cytoplasm (e.g., in *Saccharomyces cerevisiae, Plasmodium sp.*) (Mowbrey & Dacks, 2009). The precise mechanism
controlling Golgi cisternae assembly is partially understood, but a role of the Golgi Reassembly and Stacking Proteins (GRASPs), located on the cisternal rims of the Golgi, for this function has been proposed (FA. et al., 1997; Shorter et al., 1999; Xiang & Wang, 2010; Zhang & Wang, 2016). Mammalian cells have two paralogue genes, GRASP55 and GRASP65 that associate with membranes of cis and medial-trans cisternae, respectively, via N-terminal myristoylation. Depletion of GRASP55 or GRASP65 reduces the number of cisternae per Golgi stack and knockdown of both genes induces the dismantling of the entire stack, resulting in acceleration in protein trafficking but impairment in accurate protein glycosylation and sorting. GRASPs form trans-oligomers through their N-terminal domain to stick adjacent cisternae and GRASP oligomerization is regulated by phosphorylation within the C-terminal serine/proline-rich domain.

GRASPs are evolutionally conserved proteins (except in plants), and even lower eukaryotes with unstacked Golgi bodies have a GRASP gene (Vinke et al., 2011), pointing to GRASPs as membrane tethers involved in processes others than Golgi stacking and architecture. In fact, it has been reported that GRASPs can associate with different organelles, displaying various cellular functions depending on their locations (Rabouille & Linstedt, 2016). For example, GRASPs at the ER contribute to the initial step of N-linked glycosylation while at ER exit sites (ERES), they are involved in the sorting and transfer of certain transmembrane proteins across the ER membrane. In addition to the classical ER>Golgi>plasma membrane secretory pathway, some proteins are delivered to the plasma membrane or the extracellular space in an unconventional manner, involving at least four different mechanisms (Ng & Tang, 2016). GRASPs are involved in a Golgi-bypass of transmembrane proteins and the secretion of cytosolic proteins to the extracellular medium by secretory autophagy or exophagy. This latter pathway requires the formation of specific autophagosomes (named compartments for unconventional protein secretion (UPS), or CUPS) near ERES, where GRASPs localize, and CUPS fusion with the plasma membrane to enable the delivery of their content to the extracellular medium (Bruns et al., 2011). In particular, GRASPs mediate the tethering of CUPS at the plasma membrane prior to fusion. Moreover, certain stimuli that
lead to changes in phosphorylation or glycosylation, redirect GRASPs from Golgi to ER, autophagosomes, or MVBs to participate in UPS (Noh et al., 2022).

*S. cerevisiae* possesses a gene orthologue for GRASP65 (Grh1) that plays a role in ER to Golgi traffic (Behnia et al., 2007). In addition to localization to dispersed tubules (Rambourg et al., 2001), Grh1 is also observed on CUPS for unconventional secretion of cytoplasmic proteins but although Grh1 is not essential for growth or secretion (Curwin et al., 2016). The GRASP homolog in *Dictyostelium discoideum* (GrpA) is involved in the unconventional secretion of the acyl coenzyme A binding protein, a precursor of the spore differentiation factor-2, and is required for spore viability (Kinseth et al., 2007). The fungal pathogen *Cryptococcus neoformans* has a GRASP ortholog relevant for Golgi homeostasis and required for loading into secretory vesicles the capsule-associated immune-modulatory polysaccharide glucuronoxylomannan (Kinseth et al., 2007; Peres da Silva et al., 2018). The causative agent of malaria, *Plasmodium falciparum*, expresses a GRASP gene homolog composed of 3 exons, close to mammalian GRASP55 (PfGRASP), which exists in two isoforms: one encoding for a myristoylated protein (GRASP1 with predicted MWt of 66.8-kDa) and a non-myristoylated protein (GRASP2 with a predicted MWt of 68.4-kDa) (Struck, Herrmann, Langer, et al., 2008). PfGRASP has a conserved domain structure with a N-terminal GRASP domain for dimerization and trans-oligomerization and a proline-serine-rich C-terminus. GRASP1 contains the N-terminal myristoylation motif present in higher eukaryote GRASPs while GRASP2 has fungi-like N-terminal domain with a stretch of hydrophobic amino acids that resembles a signal anchor sequence for membrane association via the acetylation of a phenylalanine residue.

In *P. falciparum* blood forms, Golgi structures are composed of dispersed, unstacked *cis*-cisternae (marked by ERD2 and PfBet3p) and *trans*-cisternae (marked by PfRab6), in close proximity to transitional ER (marked by PfSec13p) (Adisa et al., 2007; Bannister et al., 2004; Struck, Herrmann, Schmuck-Barkmann, et al., 2008; Van Wye et al., 1996). The parasite secretory pathway also extends beyond the PV with a COPII-mediated vesicle transport exported into the host cytoplasm of red blood cell (Marti et al., 2005). Localization studies on *P. falciparum* blood forms show co-distribution of the two
isoforms of PfGRASP on small punctate structures in the cytoplasm; none of these isoforms are exported
to the host cell (Struck et al., 2005; Struck, Herrmann, Langer, et al., 2008). Immunolocalization assays
using Golgi markers reveals that PfGRASP distributes to cis-Golgi vesicles, very close to vesicles
positive for PfRab6. Abrogation of the glycine residue for myristoylation in GRASP1 or deletion of the
N-terminus of GRASP2 results in accumulation of each isoform in the parasite cytosol (Struck et al.,
2005; Struck, Herrmann, Schmuck-Barkmann, et al., 2008).

The function of GRASP has never been investigated in Plasmodium parasites during development
in host of mosquito vector, but some hypotheses have been proposed related to organelle tethering. P.
falciparum contains a large endosomal compartment positive for PfRab7 that plays a role in protein
trafficking (Krai et al., 2014). This endosome resides in close proximity to cis- and trans-Golgi cisterna
and transitional ER sites where the cargo is organized for delivery to organelles or export (Lee et al.,
2008). A tethering mechanism may hold these organelles close to each other to limit the traveling distance
of coated vesicles with COPII, COPI or clathrin. It is plausible that PfGRASP on cis-Golgi vesicles act as
a tether protein to allow effective protein transport between secretory organelles. A microscopy study on
transgenic Plasmodium berghei parasites expressing fluorescently tagged GRASP to explore the
distribution of the ER and Golgi structures in liver forms illustrate GRASP foci close to patches of ER
accumulated in the cytoplasm of trophozoites and schizonts (Kaiser et al., 2016). Similarly, GRASP is
perhaps involved to maintain the ER close the Golgi vesicles (Kaiser et al., 2016). Later on, when the
plasma membrane of schizonts invaginates at the cytomere stage to partition the cytoplasm in view of
merozoite individualization (Graewe et al., 2012), GRASP puncta redistribute at the parasite periphery,
underlying the plasma membrane away from ER patches; each nascent merozoite inherit a single GRASP
vesicle localized at a distinct site of the perinuclear ER. Ultrastructural studies on P. berghei sporozoites
developing in mosquitoes show a group of 1 to 3 smooth cisternae parallel to each other that are close to a
zone of coated vesicles budding from the nuclear envelope, suggestive of Golgi properties (Schrevel et
al., 2008). Although there is no evidence that GRASP is expressed on these cisternae, such a protein may
contribute to stack these Golgi structures, like for mammalian Golgi cisternae.
We previously documented that *Plasmodium* sporozoites transition to replication-competent trophozoites in hepatocytes by dismantling the cytoskeleton and the inner membrane complex, and by discarding invasion organelles, such as secretory micronemes (Jayabalasingham et al., 2010). In sporozoites, micronemes are ovoid organelles of 20-25-nm individually dispersed throughout the cytoplasm; during sporozoite-to-trophozoite conversion, micronemes cluster and are sequestered in double-membrane sac expressing ATG8 and originating from the endosymbiotic organelle, the apicoplast (Voss et al., 2016). Upon closure, microneme-containing autophagosomes in *P. berghei* associated with GRASP and the vacuolar protein sorting-associated protein 4 (VPS4) to form amphisomes that fuse with the plasma membrane to release micronemes into the PV lumen for degradation.

### VI. Aim

The lab has engineered a *P. berghei* mutant lacking the GRASP gene to examine the contribution of *Plasmodium* GRASP to parasite development in the mosquito vector and mammalian host. In particular, a major part of my thesis work will focus on the metamorphosis of GRASP-KO parasites in the liver to explore our hypothesis of GRASP mediating organelle exophagy from intra-hepatic sporozoites by monitoring the localization of GRASP and its distribution relative to micronemes. In addition, my thesis will also focus on cloning the lab engineered *P. berghei* mutant lacking the VPS4 gene to understand the role of VPS4 in *Plasmodium* development.

Malaria reflects a very complex set of interactions between the parasite, the human host, and the vector responsible for transmission. Changes in morphology and metabolism are essential for *Plasmodium* parasites cycling between the host and the mosquito vector. Therefore, gene products involved in parasite metamorphosis and adaptation represent potential therapeutic targets.
I. Generation of Pb-GRASP specific antibody in mice to study the localization of the Golgi.

A. Reagents and Antibodies

All chemicals were obtained from New England BioLabs® (USA) or Bio-Rad (USA) unless otherwise stated. For western blotting, antibodies used include anti-6xHIS tag mouse monoclonal antibody (HIS.H8) (ThermoFisher Scientific, USA) and mouse anti-blood stage *P. berghei* polyclonal IgG antibody. Since all the primary antibodies used for western blotting were raised in mice, horseradish peroxidase-conjugated goat anti-mouse IgG (GE Healthcare, UK) was used as the secondary antibody. Antibodies used for the immunofluorescence assays include mouse anti-HSP70 (4C9) (diluted at 1:100), a generous gift from Prof. Fidel Zavala (Johns Hopkins University), rabbit anti-TRAP (diluted 1:200) kindly provided by Prof. Photini Sinnis and goat anti-UIS4 (LSBio, USA) (diluted 1:300). The DAPI staining for all cells was performed at a dilution of 1:1000 with a concentration of 1 µg/ml. Secondary antibodies used for immunofluorescence assays include: anti-mouse (Alexa-488), anti-rabbit (Alexa-647), and anti-goat (Alexa-568).

B. Mammalian cell lines, bacterial cells and parasite strains

The transformed *E. coli* cells used here are One Shot™ TOP10 Chemically Competent *E. coli* (ThermoFisher, USA), Chemically Competent M15 (pREP4) *E. coli* and Chemically Competent SG13009 (pREP4) *E. coli*. The TOP10 *E. coli*, after transformation with appropriate plasmid, were grown in LB medium containing 100 µg/ml Ampicillin. The transformed M15 (pREP4) *E. coli* and SG13009 (pREP4) *E. coli* were grown in LB medium containing 100 µg/ml Ampicillin and 50 µg/ml Kanamycin.
The commercial mammalian cell line mouse Hepa1-6 (ATCC, CRL-1830) was used in this study for its monolayer growth pattern. The cells were grown in medium consisting of αMinimum essential medium (αMEM) with 10% Fetal Bovine Serum (FBS), 2 mM of L-Glutamine, 100 U/ml of Penicillin-Streptomycin and 0.25 μg/ml Fungizone at 37°C in an atmosphere of 5% CO2.

The *Plasmodium berghei* ANKA strain was selected as the background strain for our study due to the use of previously generated mutants ATG8 Over Expressor (PbATG8-OE) and GRASP Knock Out (PbGRASP-KO). The PbGRASP-KO was used to perform comparative studies of PbGRASP with the wild type.

C. In vivo malaria model

All procedures were performed using permits issued to Animal Care and Use Committee of Bloomberg School of Public Health, Johns Hopkins University for protocol # MO19H282. Naïve, female BALB/c mice were chosen as the rodent model for all the experiments performed below. All mouse dissections were performed under anesthesia. The mice were anesthetized with 200 µl of Ketamine per mouse (ketamine 100 mg/ml, and prochlorperazine (Acepromazine) 10 mg/ml in 0.9% saline).

D. *Plasmodium* sporozoite cultivation

Salivary glands were dissected from infected *Anopheles stephensi* mosquitoes between 21 and 26 days p.i. and stored on ice in 1X Phosphate-buffered saline (PBS). The salivary glands were disrupted using a 27.5-gauge needle to release *P. berghei* sporozoites, which were then diluted in 1X PBS for *in vitro* infection. For infection of Hepa1-6 cells, dissected salivary glands were stored in 1X PBS on ice. The released sporozoites were treated in αMEM with 100 U/ml of Penicillin-Streptomycin (PS), and 0.25 μg/ml of Fungizone before counting using a C-Chip hemocytometer.

E. Cloning

To generate *Pb*-GRASP specific antibodies to study the localization of GRASP during the *Plasmodium* development, a multi-epitope synthetic *Pb*GRASP gene (synGRASP gene) was designed by
Dr. Tejram Sahu from Johns Hopkins University. The immunogenic sites from *Pb*-GRASP were identified using the AbDesigner software (Pisitkun et al., 2012) and stitched together through triple glycine sequences between the restriction sites of BamHI and HindIII (Figure 3). The 273 base pair long synGRASP gene was generated with the help of Integrated DNA Technologies, in a pDEF plasmid.

The bacterial expression plasmid pQE30, suitable for expression in M15 or BL21-DE3 bacterial cells, was selected for cloning synGRASP gene (Figure 4A). The presence of selection marker, AmpR and the poorly immunogenic 6xHis tag also facilitated cloning and purification respectively. The AmpR confers resistance to Ampicillin helping with the selection of transformed clones. The 6xHis tag’s high affinity to Ni-NTA facilitates purification and acts as a primary antibody target during western blot. The pQE30 (vector) and the synGRASP gene (insert) were prepared individually using double restriction digestion at the restriction sites of enzymes BamHI and HindIII. About 3 µg of pQE30 or synGRASP gene in pDEF were used to set up a 50 µl reaction mixture with 5 µl of CutSmart® 10X Buffer, 3 µl of BamHI-HF®, 3 µl of HindIII-HF® and Nuclease-free water. The restriction digestion was performed by incubating the reaction mixture at 37°C for 1h followed by heat inactivation of HindIII enzyme at 80°C for 20 minutes (min) and stored at 4°C.

The size of the digested vector and insert were verified through electrophoresis on a 1% agarose gel. All the samples loaded in agarose gel are mixed 5:1 with 6X Gel Loading Dye Purple. A 1kB DNA Ladder and a 1kB Plus (2-Log) DNA Ladder were used to verify the 273 bp and 3.4 kb size of synGRASP gene and pQE30 vector respectively. The gel was stained in ethidium bromide and imaged using UV light.

The synGRASP gene insert was cut from the gel and purified using Monarch® DNA Gel Extraction Kit. Additionally, the vector was also cleaned using QIAquick Nucleotide Removal kit. The vector and insert were ligated in the ratio of 1:3 using Thermo Scientific™ Rapid DNA Ligation Kit with a reaction set up for vector-only as a negative control (Figure 4C). Four microliters of 5X Rapid Ligation Buffer and one microliter of T4 DNA Ligase were added to the vector-insert ratio and vector-only tubes
supplemented by nuclease-free water to make up the volume to 20 µl. The ligation reaction proceeded at 20°C for 30 min, remaining at 4°C until transformation.

F. Transformation

Three microliters of the recombinant plasmid (pQE30-synGRASP) ligation mixture were added to One Shot™ TOP10 Chemically Competent *E. coli* cells and incubated on ice for 30 min. The cells were subjected to heat shock at 42°C for 30 sec in a water bath and placed in ice water immediately for 5 min. Next, 250 µl of SOC media was added and the cells were incubated at 37°C for 1h with continuous shaking at 225 rpm in a MULTITRON® version 2 (INFORS HT, USA). The transformed *E. coli* cells (~150 µl) were plated on LB. agarose plates with 100 µg/ml Ampicillin and incubated for 16-18h at 37°C. The pQE30 was also transformed into TOP10 *E. coli* cells to act as vector-control. Nine clones were screened using vacuum-based plasmid minipreps and restriction digestion using appropriate restriction enzymes with a clone from vector-only used as a negative control. Restriction positive clones were identified through 1% agarose gel electrophoresis and sequenced to confirm the presence of the synGRASP gene. The successfully transformed *E. coli* clones were preserved with a ratio of 1:1 of bacterial cell culture and 40% glycerol at -80°C.

The transformed TOP10 *E. coli* cells are not competent to regulate gene expression. Hence, the recombinant plasmid was transformed into Chemically Competent SG13009 and M15 *E. coli* cells, containing the pREP4 plasmid. The protein synthesis is efficiently regulated and repressed by the lac repressor expressed by the pREP4 plasmid. pREP4 also confers Kanamycin resistance. Hundred nanograms of recombinant plasmid was transformed into M15 and SG13009 cells and plated (~80 µl) on LB. agarose plates with 100 µg/ml Ampicillin and 50 µg/ml Kanamycin and incubated for 16-18h at 37°C. However, during the transformation, the cells were subjected to a longer heat-shock of 90 sec. Five colonies each from transformed M15 and SG13009 cells were screened using vacuum-based plasmid minipreps and restriction digestion using appropriate restriction enzymes with one clone each from
vector-only transformed cells as negative control. All the clones from recombinant plasmid were identified to be positively transformed using 1% agarose gel.

G. Gene Expression

To induce gene expression in transformed SG13009 and M15 cells, one clone each was chosen to be streaked on LB. agarose plates with 100 µg/ml Ampicillin and 50 µg/ml Kanamycin and incubated for 16-18h at 37°C. Single colonies from these transformants was picked into 1.5 ml LB. broth containing 100 µg/ml Ampicillin and 50 µg/ml Kanamycin and incubated for 16-18h at 37°C with continuous shaking at 225 rpm. Simultaneously, two 1.5 ml cultures were inoculated with a colony each of pQE30 transformed SG13009 and M15 cells as control. Five Hundred microliters of the overnight culture were inoculated into 10 mL of prewarmed media containing appropriate antibiotics at 37°C for 30 min with vigorous shaking. The cultures were incubated for another 45 min till they reached an OD600 value between 0.5 – 0.7. Before inducing expression, 1 ml from each culture was removed to serve as non-induced control. To the remaining culture volume, IPTG was added to a final concentration of 1 mM and incubated for 4h at 37°C with vigorous shaking. The cells were harvested by centrifuging them for 1 min at 15,000 x g. After discarding the supernatant, the non-induced and induced cell pellets were stored at -20°C till further experiments.

Based on pellet size, the pellets were resuspended in equal volumes of 1X PBS and 2X Laemmli Sample Buffer containing 5% β-mercaptoethanol, sequentially. The final solution was boiled for 10 min at 100°C using a heat block and centrifuged at 16,000 x g for 5 min. Ten microliters of supernatant was used to load 15% Sodium Dodecyl Sulphate-PAGE gels with Precision Plus Protein™ Kaleidoscope™ Prestained Protein Standards marker was used. The gels were run in 10X Tris/Glycine/SDS Buffer diluted 1:10 in distilled water.

Two gels were loaded, one for Coomassie and the other for western blot, and run at 80 V for 15-20 min until the proteins crossed the stacking gel and then at 100 V till completion. One gel was left in
Coomassie stain, made of destaining solution mixed with 50 mg/100 ml Coomassie Brilliant Blue R-250, on a rocking platform overnight. The gel was then transferred to the destaining solution, for 1-2 days to remove the Coomassie stain from non-proteins, and finally left in distilled water until imaging. The destaining solution has a composition of 10% acetic acid and 40% ethanol in distilled water.

The second gel was used to set up protein transfer onto a nitrocellulose membrane in 1X transfer buffer - distilled water with 20% methanol and 25X Tris-Glycine Transfer Buffer, added sequentially. Before packing the gel and membrane between the sponges and the blotting paper, the gel and membrane were rinsed in transfer buffer for 20 min and 15 min respectively. The protein transfer was run for 16-18h at 16 V, at 4°C.

The membrane was blocked for 1h using 4% skimmed milk in 1X TBS-T, containing 20 µl Tween per 50 ml 1X TBS, on a rocking platform. Next, it was stained 1/10000 dilution of mouse anti-His monoclonal antibody in 3% Skimmed milk in 1X TBS-T and left overnight at 4°C. The next day, after washing the membrane thrice with TBS-T for 5 minutes, it was stained with 1/10000 dilution of secondary antibody, anti-mouse Horseradish Peroxidase (HRP), in 1X TBS-T for 1h. Next, it was washed 4-5X with TBS-T for 5 min and lastly with TBS for 5 min. The membrane was incubated for 5 min with 2 ml Clarity™ Western ECL Substrate in the dark and imaged using C-Digit® Blot Scanner (LI-COR, USA) and Image Studio™ software. After verifying the expression, the clones were preserved with a ratio of 1:1 of bacterial cell culture and 40% glycerol at -80°C.

Due to similar levels of expression in both M15 and SG13009 cells, as seen from imaging, M15 E. coli cells were chosen for further expression optimization and purification.

H. Temperature Optimization for Expression and Target Protein Solubility

In a 50 ml flask, a colony from pQE30-synGRASP transformed M15 cells was inoculated in 10 ml LB broth with 100 µg/ml Ampicillin and 50 µg/ml Kanamycin and grown at 37°C with vigorous shaking. For temperature optimization, 2.5 ml of the culture was inoculated in 50 mL LB media with
appropriate antibiotics at 25°C, 30°C and 37°C for 3h, 2h and 75 min respectively till an OD600 value of 0.5-0.7 was reached. The expression was induced using IPTG and the cultures were grown for 4h at 37°C and for 6hrs at 30°C or 25°C. One ml volume for non-induced and induced control were taken at appropriate times and pelletized before storing them at -20°C. The cells were harvested by centrifugation at 4000 x g for 20 min.

The pellets were weighed after discarding the supernatant and resuspended in 1 ml Lysis buffer per 0.1 g pellet. The Lysis buffer was made using 50 mM NaH2PO4, 300 mM NaCl and 10 mM imidazole with pH adjusted to 8.0 using NaOH. To the resuspended cells, lysozyme was added to 1mg/ml and incubated on ice for 30 min. Keeping it on ice, the lysate was subjected to sonication for 6 x 10 sec with 10 sec pauses at 200-300 W using Branson Ultrasonics™ Sonifier® S-450A (Emerson, USA) equipped with a microtip. The lysate was then centrifuged at 10,000 x g at 4°C for 20-30 min. After separating the supernatant that contains the soluble fraction of protein, the pellet was resuspended in 5 ml lysis buffer which forms the insoluble fraction of protein. The non-induced control and induced control pellets were resuspended in 1X PBS. The samples were prepared accordingly, 1:1 in 2X Sample Buffer, and run in 15% SDS-PAGE gels. The protein expression across the different temperatures (25°C, 30°C, 37°C) and solubility was visualized using Coomassie stain and Western Blot using the anti-6xHisTag Antibody.

I. Protein Purification

For purification under native conditions, 20 ml of LB broth containing 100 µg/ml Ampicillin and 50 µg/ml Kanamycin was inoculated with a colony of transformed M15 cells and grown at 37°C overnight with vigorous shaking. The non-induced culture was inoculated 1:50 in four 1 L sterile conical flasks each containing 250 ml of LB media, containing appropriate antibiotics. The culture was allowed to grow at 37°C for 95 min until an OD600 value of 0.6 is reached. After inducing expression using IPTG, the cells were incubated at 37°C for 4h with vigorous shaking. The cells were harvested by centrifugation.
at 4000 x g for 20 min and weighed before storing the pellet at -20°C for lysis. The non-induced and induced control were also prepared.

The next day, the cell pellet was thawed on ice for 15 min and resuspended in lysis buffer at 3-5 ml per gram wet weight. To this, lysozyme to a final concentration was added and incubated on ice for 30 min. Using a microtip, the sample was sonicated for 6 x 10 sec with 10 sec pauses at 200-300W on ice. The lysate was centrifuged at 10,000 x g at 4°C for 30 min and the supernatant was saved for purification under native conditions.

For batch purification of 6xHis-tagged synthetic GRASP protein (synGRASP) from *E. coli* under native conditions, 8 ml of Ni-NTA agarose (Qiagen, USA) was spun down for 3 min at 1,500 x g in and washed with 4 ml lysis buffer. The cleared lysate was mixed 4:1 to the Ni-NTA slurry and left to bind overnight on a rotary shaker at 200 rpm and 4°C. The next day, the lysate-Ni-NTA mixture was transferred to a capped column for further purification at 4°C. The bottom cap was removed to collect the flow through. The beads were then washed thrice with 4 ml Wash buffer and the wash fractions were collected separately. The Wash buffer was made of 50 mM NaH2PO4, 300 mM NaCl and 50 mM imidazole with an 8.0 pH adjusted using NaOH. Finally, the protein was eluted 6x and 4x using 0.5 ml and 1ml Elution buffer respectively in separate tubes. The Elution buffer is made of 50 mM NaH2PO4, 300 mM NaCl and 250 mM imidazole, and its pH adjusted to 8.0 using NaOH. The flow through, 3 wash fractions and 6 eluates were analyzed using Coomassie and western blot.

A second purification method was used to optimize the purification of the synthetic PbGRASP protein. A new 1 L culture of transformed M15 cells was inoculated, induced and lysed to get the supernatant as mentioned above. One milliliter each were stored for a non-induced control and lysate control at appropriate times. The supernatant was purified using Fast Protein Liquid Chromatography (FPLC) with the help of Dr. Abhishek Gupta from Johns Hopkins University. The supernatant was loaded into an AKTA pure 25 M FPLC system (GE Healthcare/Cytiva) and applied to a 5 mL HisTrap HP column (GE Healthcare/Cytiva). The column was washed with 10 column volumes of protein binding
buffer (1 M KCl, 1 M Urea, 50 mM Tris-HCl pH 7.4, 10 mM imidazole, 1.5 mM β-mercaptoethanol, 5% (v/v) glycerol). Protein was eluted by linearly increasing the imidazole concentration with Protein Elution Buffer (1 M KCl, 1 M Urea, 50 mM Tris-HCl pH 7.4, 500 mM imidazole, 1.5 mM β-mercaptoethanol). The fractions containing substantial protein were stored in protein elution buffer with 25% glycerol at 4°C. The HisTrap HP column was stripped every 5-10 purifications by incubating in Column Stripping Buffer (500 mM NaCl, 50 mM EDTA, pH 8.0, 20 mM Na$_3$PO$_4$, pH 7.0) for 20 min, then in 1 M NaOH for 2 h. Nickel (100 mM NiSO$_4$) was flowed through the column at 0.5 mL/min for 50 min to regenerate the HisTrap HP column. The non-induced control, lysate control and eluates 14 – 19 were analyzed using Coomassie and western blot to identify eluates containing the target protein, named synGRASP-U (U for urea in Protein Elution Buffer).

Since the elution buffer used in purifying synGRASP-U protein contains urea, a denaturing agent, the buffer was exchanged using dialysis. The isoelectric point of the synthetic GRASP protein sequence was computationally predicted using Isoelectric Point Calculator 2.0 (Kozlowski, 2021). The buffer was exchanged to PBS using Slide-A-Lyzer™ Dialysis Kit to give synGRASP-P protein (P for protein buffer PBS).

J. Protein Quantification

The protein concentration in eluates of synGRASP, synGRASP-U and synGRASP-P was quantified using Pierce™ BCA Protein Assay Kit, compatible with proteins eluted using imidazole. The chosen eluates were mixed to form one protein solution per purification method. The BSA standards were diluted with appropriate elution buffers based on the protein being measured. The standards and samples were loaded in a 96-well round bottom microplate and quantified using SoftMax® Pro 7.0 software (Table 1).
K. Antibody generation through mice immunization

As illustrated in Figure 5, mice were immunized to generate antibodies against the synthetic GRASP proteins. To obtain pre-immune sera, 100-200 µl of blood was collected in 1.5ml Eppendorf tubes from six mice through retro orbital bleeding one week before immunization. The blood was stored at 4°C overnight for the cells to separate from the sera. The tubes were centrifuged at 1000 x g for 5 min at room temperature (RT) and the serum was collected and stored at -20°C.

The first dose of immunization was prepared using the three different strategies with synGRASP, synGRASP-U and synGRASP-P. For each dose, 20-40 µg of protein was injected intraperitonially or intradermally to the mice. The first dose was mixed with Freund’s Adjuvant, Complete (Sigma) and was supplemented with a booster dose given 2 weeks later with Freund's Adjuvant, Incomplete (Sigma). Two 2.5 ml Gastight Instrument Syringes, 1002 TLL (Hamilton), connected using a Discofix® 3-Way Stopcock (B|BRAUN,USA), was used to make the 1:1 peptide-adjuvant oil emulsion.

For the first strategy, protein synGRASP was run through SDS-PAGE gel and stained in Coomassie. After removing the stain using destaining solution and distilled water, the bands containing the synGRASP peptide were cut from the gel and stored in PBS at 4°C. The gel pieces were crushed using 28 ½ gauge syringes before mixing with the adjuvant. For the second strategy, the synGRASP-U peptide was used to prepare the first dose. The final strategy and the booster dose were made using synGRASP-P.

Two weeks post the booster dose, blood was collected from immunized mice through cardiac puncture and kept at RT for 15-20 min for the serum to separate. Then, it was centrifuged at 1000 x g for 10 min at 4°C before aliquoting the serum and storing at -20°C.

L. Measuring Antibody Activity

The antibody activity was first tested against the purified synthetic GRASP protein. Ten microliters of neat and 1:5 dilution of synGRASP-P samples prepared appropriately were loaded in SDS-
PAGE gels for western blotting. *Plasmodium berghei* extract was prepared by incubating infected blood with 1:2 of 0.2% Saponin solution on ice for 4-5 min. After centrifuging the cells for 5 min at 10,000 rpm and 4°C, the pellet was washed 3x with 1ml of 1X PBS for 5 min at 3000 rpm and RT. The resulting pellet was resuspended in 1X PBS and prepared for SDS-PAGE electrophoresis.

To compare the antibody activity, anti-His antibody, pre-immune sera, anti-synGRASP antibody, anti-synGRASP-U antibody and anti-synGRASP-P antibody (1:200) were used as the primary antibody. Since, all the primary antibodies are raised in mice, anti-mice HRP antibody was used as the secondary antibody.

The antibody activity of anti-synGRASP-P antibody was then tested against the blood-stage parasite lysate of WT *Plasmodium berghei* and increasing volumes of the PbGRASP-KO, studied extensively in the lab by Dr Tejram Sahu and Ella Gehrke. After the saponin lysis and 1X PBS washes of the infected blood, the parasite lysates were prepared by resuspending the pellet in 50 µl of Passive Lysis 5X Buffer (Promega). The suspensions were incubated at RT for 15 min and centrifuged at 16,000 x g for 5 min at 4°C. The supernatants, containing the WT or PbGRASP-KO lysate, were stored at -20°C and used for western blot. The anti-*Pb* Blood Stage antibody (control) and anti-synGRASP-P antibody were used in 1:500 dilutions as primary antibodies with anti-mice HRP antibody as the secondary antibody.

M. Development of Liver Stages *in vitro*

One milliliter of Hepa1-6 cells was added to a 15 ml falcon tube with 5 ml of cell medium (αMEM with 10% FBS, 2 mM of L-Glutamine, 100 U/ml of Penicillin-Streptomycin, 0.25 µg/ml Fungizone). Cells were spun at 1,500 rpm (Backman Coulter Allegra 6r centrifuge - GH-3.8 rotor). After vacuuming the supernatant to remove the cryoprotectant DMSO from the previous cell preservation steps, the cell pellet was resuspended in 1.5 ml of cell medium. The cells were added to a T25 flask and incubated at 37°C with cell medium changed every 24h. At 80% confluence, Hepa1-6 cells were washed twice with 5 ml of prewarmed 1X PBS. Next, 1 ml of Trypsin was added to the cell layer and vacuumed
out. The flask was then incubated with 3 ml of Trypsin at 37°C for 3-5 min. To stop the trypsin digestion, 9 ml of cell medium was added. The resulting cell mixture was transferred to a 15 ml falcon tube and spun at 2,000 rpm (Backman Coulter Allegra 6r centrifuge - GH-3.8 rotor) for 5 min. Post centrifugation, the supernatant was vacuumed out and the cell pellet was resuspended in 5 ml of cell medium. To seed the cells for in vitro infection, the cells were quantified using a 1:5 dilution of resuspended cells in Trypan blue. The cells were seeded in a 24-well plate at the dilution of 100,000 cells per well, infected with 20,000 sporozoites and spun at 2,000 rpm (Backman Coulter Allegra 6r centrifuge - GH-3.8 rotor) for 5 min. The infection was prolonged for 3h at 37°C prior to washing 3-times with 1X PBS subsidized with 100 U/mL of PS and 0.25 μg/mL of Fungizone and returned to the 37°C incubator. Cells were fixed at different time points for IFA or DAPI staining. For plates fixed past 24h p.i., cell medium was changed every 24h, subsidizes with 0.12 μg/ml of Mycamine.

N.  Immunofluorescence Assays

The wells with infected Hepa1-6 cells were washed with PBS, fixed with 250 μl of fixative solution (5 ml water, 1 ml of 10X PBS, 4 ml 10% formaldehyde, 8 μl 27% glutaraldehyde) for 15 min at RT, permeabilized with 0.3% Triton X-100 in 1X PBS at RT for 6 min, blocked with 3% BSA in 1X PBS for 1h or overnight at 4°C. All the steps were carried out while keeping the 24-well plates covered in aluminum foil to minimize light exposure.

To study the detection activity of antibody generated against PbGRASP, cells fixed at 24h were exposed to mouse anti-synGRASP-P antibody and goat anti-UIS4 in 3% BSA in 1X PBS. Following a 2h incubation at RT, the wells were left at 4°C overnight. After washing the wells three times with 1X PBS, the wells were exposed to secondary antibodies coupled to Alexa as described (Table 2). DAPI stock was diluted to 1 μg/mL and added to wells at a 1:1000 dilution for 5 min, followed by three wash steps with 1X PBS. Coverslips were removed and rinsed in 1X PBS and water before mounting onto a slide with 3 μl of ProLong Diamond antifade mounting solution.
To study the morphological differences between PbGRASP-KO and WT, two sets of experiments were run. First, the cells fixed at 24h, 48h and 67h for PV enumeration in EEF and to obtain data on average PV volume. In addition to obtaining data on average PV volume, cells were fixed at 4h, 10h, 22h and 36h to quantify the TRAP signal in the parasite cytosol. The blocked cells were exposed to primary antibodies mouse anti-HSP70 (4C9), rabbit anti-TRAP, goat anti-UIS4 in 3% BSA in 1X PBS. Following the incubation with the primary antibodies, the wells were exposed to appropriate secondary antibodies (Table 2) and stained with DAPI before mounting the coverslips onto a slide with 3 µl of ProLong Diamond antifade mounting solution.

Infected cells were visualized with a Zeiss AxioImager M2 fluorescence microscope equipped with an oil-immersion Zeiss plan Apo 100x/NA1.4 objective and a Hamamatsu ORCA-R2 camera. Optical Z-sections with a 0.2 µm spacing were captured using the Volocity software (Quorum Technologies, Puslinch, ON, Canada). Images of PV (n=26-77) were deconvolved with an iterative restoration algorithm using calculated point-spread functions, a confidence limit of 100% and a limit of 30 iterations. Images were cropped and adjusted for brightness contrast using Volocity software. Using the Volocity software, deconvolved images showing HSP70 fluorescence were used to obtain data on the average volume, measure of TRAP compartments within the HSP70 compartment and Factor Shape (with value of 1 being a perfect sphere) of WT and PbGRASP-KO parasites.

**Image Analysis**

To characterize the volume of the HSP70 compartment, we generated a measurement protocol in Volocity. The measurement protocol measured objects in the 3D-reconstructed volumes of optical z-slices. For the 4h, 10h and 22h time points, the HSP70 compartment was identified using a find objects task in the Volocity software; objects were identified by fluorescence intensity with a SD intensity thresholding step with a lower limit of 1, a minimum object size of 0.8 µm³, and a close function with four iterations. For the 36h time point, the HSP70 compartment was identified using the find objects task.
with automatic thresholding and a minimum object size of 0.8 µm³. The volume and shape factor of the HSP70 compartment were calculated. The shape factor numerically indicates how close a 3D shape is to a perfect sphere; a factor of 1 is a perfect sphere with smaller numbers indicating more irregular shapes.

To measure the number and volume of TRAP compartments in the HSP70 compartment, the HSP70 compartment was identified as described above. Then, TRAP compartments were identified using the find objects task in the Volocity software; objects were identified by fluorescence intensity with a SD intensity thresholding step with a lower limit of 3 and a minimum object size of 0.05 µm³. A separate touching objects tasks was also applied with an object size guide of 0.2 µm³. Lastly, a compartmentalize task was added where subpopulations of TRAP were identified inside (22h and 36h) or overlapping (10h) the HSP70 compartment. For the 10h time point, the overlapping option was used because for most HSP70 compartments there was a large TRAP object inside the HSP70 compartment that stretched towards the edge of the compartment and was not identified with the inside option. To measure the volume of TRAP objects between the HSP70 compartment and the PV, identified by UIS4 staining, we used the same protocol as just described except that instead of using the compartmentalize task, the subtract task was used to subtract the HSP70 object from the TRAP objects.

**Statistical analysis**

Numerical data are presented in dot plots with means indicated (GraphPad Prism). Using GraphPad Prism software, samples were compared using either a one-way ANOVA with a Tukey’s multiple comparisons test or unpaired two-tailed t-tests.
II. Cloning *Plasmodium berghei* VPS4-KO in Swiss-Webster Mice

A. Parasite strains

*Plasmodium berghei* ANKA was selected as the background strain for this study due to the use of previously generated mutants ATG8 Over Expressor (PbATG8-OE) and GRASP knockout (PbGRASP-KO). Generation of PbVPS4-KO under the same strain can be used to generate information regarding the relationship between *Pb*VPS4, *Pb*GRASP and *Pb*ATG8 in the Plasmodium secretory autophagy pathway.

B. In vivo malaria model

All procedures were performed using permits issued to Animal Care and Use Committee of Bloomberg School of Public Health, Johns Hopkins University for protocol # MO19H282. Naïve, Swiss Webster female mice were chosen as the rodent model for all the experiments performed below. All mouse dissections were performed under anesthesia. The mice were anesthetized with 200 µl of Ketamine per mouse (ketamine 100 mg/ml, and prochlorperazine (Acepromazine) 10 mg/ml in 0.9% saline).

C. Cloning

To assess the role of *Pb*VPS4 in the development of *Plasmodium* parasite, a VPS4 knockout (*Pb*VPS4-KO) was generated by Dawlyn Garcia, a previous Master of Science student at Dr. Isabelle Coppens’ lab, Johns Hopkins University. The mutants contain a selection marker, human dihydrofolate reductase (hDHFR) that confers resistance against pyrimethamine drug to the transfected parasites. The *Pb*VPS4-KO construct was generated by replacing the VPS4 coding sequence with a hDHFR selection marker using double-homologous recombination (Figure 6).

Old Protocol

The mice were infected with pyrimethamine-treated *Pb*VPS4-KO transfected schizonts taken from liquid nitrogen. The infection was allowed to progress for 4-5 days till a parasitemia of 0.1-0.5%
was achieved. To calculate the parasitemia, the percent of parasite-infected red blood cells (iRBC) per total red blood cells (RBC) were measured using giemsa-stained blood smears.

On the day of cloning, 5 µl of blood drawn from a donor mouse was added to a 1.5 ml Eppendorf tube containing 995 µl of PBS + 300 U/ml of heparin to calculate hematocrit. The solution was mixed thoroughly to prevent any clot formation and kept on ice till counting. Ten microliters of the solution were added to 2-chip Disposable Hemocytometer (Bulldog-Bio) and viewed at 40x magnification under the Zeiss AxioStar Plus Binocular microscope. A Ph2 phase was used to distinguish between the live and dead cells. In the center of 3x3 grid of the chip, that was divided into a smaller 5x5 grid, the four corners and the center grid of the smaller 5x5 grid was used to count the hematocytes. The hematocyte density in a milliliter volume was calculated by multiplying the average number of RBCs with the total number of grids, the dilution factor (200) and 10⁴. The final parasite-infected red blood cells (iRBC) density is the product of parasitemia and RBC density.

The fresh blood from donor mouse was serially diluted in 1X PBS with 300 U/ml heparin till a final concentration of 5 iRBC/ml. To clone the VPS4-KO, 50 mice were injected with 200 µl of the diluted blood containing 0.5 – 1 iRBC per mouse. The infection was allowed to grow to develop blood stage mutant clones until day 7-8 p.i. and the infection was checked using giemsa-stained blood smears. The blood was collected from the 5 positive mice through cardiac stick at 5-8% parasitemia. About 200-300 µl of the blood was stored at -20°C to extract gDNA for PCR verification.

To extract gDNA, whole blood was incubated with 0.2% Saponin, in 1:2 ratio, on ice for 5 min. The sample was centrifuged at 5900 x g for 5 min at RT. The supernatant was discarded, and the cells were washed 3x with 1 ml 1X PBS. The pellet was resuspended in 200 µl of 1X PBS and the gDNA was prepared using QIAamp DNA Blood Mini Kit.

A PCR reaction for the gDNA was set up using primers previously designed by Dawlyn to identify clonal populations of PbVPS4-KO (Table 3). Primers 1 and 2 were used to amplify 2240 bp
region overlapping the 5’ UTR and the hDHFR cassette (Figure 6). Primers 3 and 4 were used to amplify 1360 bp region overlapping the hDHFR cassette and the 3’ UTR in VPS4-KO (Figure 6).

The PCR solution contained 50% CloneAmp™ HiFi PCR Premix (Takara), 0.5-1 µl of each primer and 100-1000 ng gDNA template per reaction. For PCR reactions set up using primer pairs 1/2 and 3/4, the thermocycler was set up as follows – initial denaturation at 98°C for 5 min, followed by 34 cycles of 98°C for 30 sec, 50°C for 60 sec and 72°C for 3 min. A final extension cycle of 72°C was performed for 7 min and samples were stored at 4°C. The size of PCR products was analyzed by running them through a 1% agarose gel containing 0.1% GelRed® Nuclei Acid Stain (Biotium) with a log2 DNA ladder.

New Protocol

To increase the efficiency of cloning, changes were made to the protocol above. The infection in donor mice was allowed to progress until a parasitemia of 0.5-2% was achieved, measured using blood smears. The 5µl of fresh blood for hematocrit was added to 995 µl of HBSS-FBS media and stored on ice and the hematocyte counting was done as explained above. However, dilution factor was not included in calculating hematocyte density as the same hematocrit solution was used for dilutions. The sample was serially diluted in PBS mixed with 300 U/ml of heparin and uninfected mouse blood to inject 10 mice with 200 µl such that each mouse received 1-2 iRBCs. The mice were checked for infection beginning from day 7 and blood was collected at 5-8% parasitemia from the 5 mice that tested positive. The blood was preserved as stocks using 1:1:3 ratio for infected blood, Alsever’s solution (Sigma) and 30% glycerol (at 4°C) and stored in liquid nitrogen. The gDNA was extracted and PCR amplified using primer pairs 1/2 and 3/4 to verify presence of hDHFR cassette. To address issues arisen during PCR amplification with the primer annealing temperatures, a gradient of temperatures running from 51°C to 64°C were used for optimization. A new PCR was set up with an annealing temperature of 55°C, keeping everything else same. The potential VPS4-KO clones were identified from this PCR and sequenced.
For sequencing the gDNA, the PCR was set up with primers 1 and 4 to amplify the region containing the 5’UTR, hDHFR cassette/PbVPS4 gene and 3’UTR in PbVPS4-KO. The only difference in the thermocycler set up from before is the 34 cycles ran at 98°C for 30 sec, 54°C for 60 sec and 72°C for 5 min. The samples were stored at 4°C until sequencing.

The sequencing was performed by the Genetic Resources Core Facility, Johns Hopkins University that uses Sanger sequencing. The primers 1 – 4 were supplemented by new primers that were designed from the 5’ end of VPS4 gene on the reverse strand. The sequences obtained were run through BLAST to verify the absence of VPS4 gene from the clonal population.
Results

From the colocalization of ATG8, GRASP and VPS4 in the autophagic compartments (Figure 2G), we hypothesized that GRASP and VPS4 play an important role in the organellar clearance during intrahepatic development (Figure 2F). Therefore, deletions of the PbGRASP or PbVPS4 gene from the Plasmodium genome must have an observable impact on its metamorphosis in the liver.

I. Generation of Pb-GRASP specific antibody in mice to study the localization of the Golgi.

The model proposed to explain the clearance of micronemes and rhoptries by exophagy suggests the involvement of GRASP in the membrane fusion between autophagosomes and MVB for the formation of amphisomes. Thus, our goal is to study the effect of GRASP deletion on the sporozoite metamorphosis and analyze the localization of GRASP using IFA, using Plasmodium berghei as a model organism.

1. PbGRASP-KO sporozoites suffer from mid-stage developmental defects at 24h p.i. then their growth resume as normal rate

We conducted assays in vitro by infecting hepatic cells with WT or PbGRASP-KO (designed by Dr. Tejram Sahu and studied by Ella Gehrke and Dr. Tejram Sahu) sporozoites to analyze the PV properties of exoerythrocytic forms (EEF) over time based in immunostaining of parasites with anti-HSP70 antibody. EEF PV enumeration in Hepa1-6 cells did not show any difference between WT and KO, suggesting that the lack of GRASP has no obvious consequence on sporozoite invasion (Figure 7A). Examination of PV shape over time based on the Shape Factor (sphericity having a maximum value of 1, which corresponds to an object with a perfectly
spherical shape) measured using Volocity did not reveal any difference in the global morphology between WT and KO parasites, with lower values as expected at 4h and 10h p.i. as sporozoites undergo metamorphosis, followed by increased values at 22h in accordance with the sphericalization of trophozoites and schizonts, then decreased values as the schizont expands and need to adjust its size to the host cell interior, squeezed between organelles (Figure 7B). We monitored the PV volumetric area over time from 4h to 67h p.i. and only observed a statistically significant difference at 22h p.i. with a mean volume of 135 µm$^3$ for the KO and 175 µm$^3$ for WT (Figure 7C-E), suggesting slower development at this mid-stage time point but then schizogony resumes.

2. **No significant differences in the TRAP signal between WT and PbGRASP-KO parasites**

The distribution of TRAP-containing micronemes was visualized over time from 4h p.i. to 36h p.i. using triple IFA of WT and PbGRASP-KO with anti-PbTRAP, anti-PbUIS4 and anti-HSP70. This IFA also acts as a visual confirmation for the similar sphericity seen between WT and PbGRASP-KO across *Plasmodium* liver stage development (Figure 7B, 8, 9, 10, 11, 12). The TRAP signal starts as diffused large puncta at 4h p.i., indicative of microneme clusterization, with concentrated puncta near the nuclear region at 10h p.i. in both WT and PbGRASP-KO (Figure 8, 9). At 22h p.i., PbGRASP-KO could compartmentalize its TRAP-containing micronemes irrespective of its smaller size (Figure 7C, 10). The distribution of TRAP staining at 36h p.i. exhibits different patterns during the microneme elimination (illustrated by arrows from panels a to d/e), progressing from abundant intracytoplasmic TRAP puncta to puncta accumulating in vacuolar space with no significant differences between WT and PbGRASP-KO (Figure 11, 12). Quantification of the signal for TRAP clustered in the parasite cytosol (overlapping with the HSP70 signal) or released in the vacuolar space (beyond the HSP70 signal and within UIS4 signal) at 10, 22 and 36h p.i. did not result in any significant differences between WT and PbGRASP-KO parasites (Figure 13A-D).
3. **Successful generation of recombinant plasmid pQE30-synGRASP**

Before generating the recombinant plasmid, the size of the double digested pQE30 (vector) and the synGRASP gene (insert) were verified to be 3.4 kb and 273 bp, respectively, using a 1% agarose gel (Figure 14A). An additional well in this gel was loaded with the digested insert to be cut and purified using gel purification (Figure 14A). The purified insert was then ligated with the pQE vector to successfully produce the recombinant plasmid pQE30-synGRASP. The effective transformation of this recombinant plasmid in TOP10 *E. coli* cells resulted in two positive clones out of nine, T7 and T9, identified by the presence of synGRASP gene (Figure 14B). However, additional bands seen in T7 suggest contamination in the clone. The clone T9 was sequenced to reveal successful attachment of the synGRASP gene template in pQE30 without any mutations (Figure 14C). Hence, clone T9 was selected for further expression.

4. **M15 and SG13009 E. coli cells show similar levels of synGRASP gene expression**

From the pQE30-synGRASP transformed SG13009 and M15 cells, 5 colonies each were selected and screened using restriction digestion. All the clones, except the controls, revealed to contain a 273 bp long synGRASP gene through 1% agarose gel (Figure 15A). The clones S5 and M1 from transformed SG13009 and M15 cells, respectively, were cultured and the expression was induced by IPTG. Through Coomassie staining, it was hard to judge if the target protein was being expressed because there were no significant differences seen between the non-induced and induced wells of pQE30-synGRASP for both M15 and SG13009 cells (Figure 15B). Nevertheless, western blots performed using the anti-His antibody successfully detect the protein expressed by ATG8-ccko and pQE30-synGRASP, all of which contain a 6x His tag (Figure 15C). Moreover, the expression levels between M15 and SG13009 cells appear to be comparable.

5. **The expression of soluble target protein by pQE30-synGRASP transformed M15 E. coli cells is optimal at 37°C**
The expression of pQE30-synGRASP in M15 cells was optimized for temperature and to find solubility of target protein. From Coomassie, the target protein is predominantly produced in its soluble form with small fractions going into the insoluble bodies (Figure 16A). This result is also replicated in the western blot performed using the anti-His antibody (Figure 16B). Furthermore, the fractions of protein in both soluble and insoluble forms are increasing with temperature with the highest at 37°C, especially seen throughout the western blot (Figure 16A-B).

6. Ni-NTA purification of synGRASP under native conditions results in additional bands of larger size

   Protein purification under native conditions using Ni-NTA beads, resulted in multiple proteins being eluted in eluates E1-E6 (Figure 17A). The detection of 6x His tag in the western blot shows that a small portion of the target protein is washed away in W3 before elution (Figure 17B). Additionally, it also confirms protein is present in all eluates with large fractions in eluates E4-E6 (Figure 17B). Although the smear seen in eluates E4-E6 with anti-His antibody detection at greater molecular weights suggest target protein is polymerizing or binding with another protein. The protein concentration in synGRASP, created by mixing eluates E4-E6, is estimated to be 6.731 mg/ml from the eleventh dilution (B6) of the sample via a BCA assay (Figure 17C).

7. Protein synGRASP-U was successfully purified by FPLC under native conditions

   A second purification of target protein under native conditions from supernatant of bacterial culture was performed using FPLC with Protein Elution buffer containing urea. From the analysis of eluates sent over, eluates E16-E19 contain the target protein, as seen from Coomassie staining or western blot (Figure 18A-B). Although anti-His recognition of small amounts of protein in E15 suggests that protein elution starts here (Figure 18B). The protein in synGRASP-U, a mixture of eluates E16-E18, was quantified to be 0.72 mg/ml from its tenth dilution (B7) using BCA assay (Figure 18C).
8. The protein synGRASP-P in PBS (pH = 7.4) buffer has been successfully generated through dialysis of synGRASP-U

The isoelectric point of the target protein was predicted \textit{in silico} to be 4.55 using IPC 2.0 (Figure 19B-C). Using dialysis, the buffer of synGRASP-U, containing denaturing agent urea, was exchanged with a basic buffer, PBS (pH = 7.4), resulting in the protein, synGRASP-P, measured to have 0.426 mg/ml using BCA assay on the protein (B5) (Figure 19D).

9. The antibodies generated against synthetic PbGRASP from immunization of BALB/c mice, through various strategies, detect synGRASP-P in western blots

Western blots were performed to analyze the ability of anti-PbGRASP antibodies to detect synGRASP-P. The pre-immune sera from mice (negative control) does not detect any protein from synGRASP-P dilutions but detects few proteins from extract of \textit{Plasmodium berghei} (Figure 20A). Conversely, the anti-His antibody (positive control) detects synGRASP-P protein but not the \textit{P. berghei} extract, as expected (Figure 20B). The anti-synGRASP antibody, anti-synGRASP-U antibody and anti-synGRASP-P antibodies, all detect synGRASP-P protein and its 1:5 dilution (Figure 20C-E). The intensity of the bands seen with PbGRASP-specific antibodies suggests that detection strength of these antibodies is similar to each other but higher than anti-His antibody (Figure 20B-E). However, lack of activity of anti-PbGRASP antibodies against proteins in blood-stage parasite extract may indicate their inability to detect native PbGRASP (Figure 20C-E).

10. The anti-GRASP-P antibody detects proteins of similar size to \textit{Plasmodium berghei} GRASP in western blots

The activity of anti-GRASP-P antibody against PbGRASP was analyzed using western blots with lystaes of wild type \textit{Plasmodium berghei} ANKA and PbGRASP-KO. Anti-blood stage
antibodies of *P. berghei*, used as a positive control, detects both WT and KO lysates (Figure 21A). Moreover, from this blot, protein bands at about 27kDa and 50kDa seem to be thinner in KO in comparison to WT lysate. With equal volumes (10 µl) of WT and KO blood stage lysates, anti-GRASP-P antibody detects proteins of three sizes, roughly 27 kDa, 55kDa and 80kDa, in WT with no protein detection in KO (Figure 21B). However, when increasing volumes (10, 20, 30 µl) of KO lysate were compared with WT (10 µl), the anti-synGRASP-P antibody does detect proteins of similar sizes in both WT and KO, at higher amounts of KO lysate (Figure 21C).

11. **No significant differences between binding of anti-synGRASP-P antibody to exoerythrocytic forms of *P. berghei* ANKA and PbGRASP-KO**

   Immunofluorescence assays were performed on hepatocytes infected with the sporozoites of WT or KO to test the activity of anti-synGRASP-P antibody in vivo. By 24h, the sporozoites have transformed into a rounded form and are identified using the parasitophorous vacuole, detected by the anti-UIS4 antibody with nucleus stained in DAPI. In WT, the anti-GRASP-P signal is not limited to the cytoplasm of *Plasmodium*, but also detects the PV (Figure 22). Moreover, there is a lot of background detection that can be seen in GRASP-KO as well, suggesting that either the antibody does not detect PbGRASP as designed or there is a cross detection although weaker in the KO.
II. Cloning *Plasmodium berghei* VPS4-KO in Swiss-Webster Mice

As we hypothesize that VPS4 may be involved in the formation of amphisomes and their fusion with the plasma membrane for organelar clearance, our goal is to clone a VPS4-KO mutant in mice to study the effect of VPS4 deletion on the sporozoite metamorphosis, using *Plasmodium berghei* as a model organism.

1. **Infection rate in mice is very low using the old protocol**

   To clone PbVPS4-KO, 50 Swiss Webster mice were injected with intravenously with one iRBC/ mouse. Four mice were identified to be positive for infection through blood smears. Analysis of PCR products, with either the 5’UTR or the 3’UTR segment flanking the drug cassette amplified, through 1% agarose gels identifies M2 and M3 to be potential clones of PbVPS4-KO. Although from 5’UTR amplification, all clones M1-M4 have bands of 2240 bp (Figure 23A), only M2 and M3 appear to contain the amplified 3’UTR of 1360 bp (Figure 23B).

2. **New protocol for cloning has led to improved outcomes for positive infections in mice**

   Ten mice were injected intravenously with 2 iRBCs/ mouse, out of which 5 mice showed infection in their blood smears. Clones C2-C4 were positive for 5’UTR of size 2240 bp but had multiple bands for the 3’UTR amplification, suggesting poor annealing of primers 3 and 4 (Figure 24A). Using a gradient for annealing temperatures, ranging from 51C to 64C, temperatures close to 55C were identified to be a good fit for primer annealing (Figure 24B). PCR amplification using 55C as annealing temperature, clones C2-C4 were reverified to contain 5’UTR and potentially bands for 3’UTR, close to 1360 bp (Figure 24C).

3. **The clonal populations obtained have parasites with PbVPS4 gene**

   The genomic region of clones C2-C4 from the 5’UTR to 3’UTR region flanking the PbVPS4 gene or hDHFR cassette was amplified using primers 1-4. The sequencing obtained from
these segments using the primers 1-4 and VPS4_Rev showed alignment with the *Plasmodium berghei* genomic segment containing the both 5’UTR and 3’UTR and the PbVPS4 gene. From sequence alignment of clones C2-C4, sequences from primer 1 and primer 4 align with the respective 5’UTR and 3’UTR region respectively (Figure 25A-C). In addition, sequences obtained from primers 2 and 3 do not align anywhere in the selected *Plasmodium* genomic region for any clone, except for clone C2 where primer 3 failed to give a sequence. However, for all these clones, sequences obtained from VPS4_Rev successfully align with PbVPS4 gene, resulting in a failure in obtaining clonal population of PbVPS4-KO (Figure 25D).
Discussion

As Plasmodium spp. cycles between its two hosts, transforming into various forms, it must adapt to different tissue environments. These adaptations are reliant on morphological and metabolic changes the parasite undergoes during its transformation. Of the multiple transitions, the sporozoite metamorphosis into liver form is of critical importance for disease propagation in host. The sporozoite transfer to the host via an infectious mosquito bite acts as a bottleneck event due to smaller numbers of sporozoites entering the skin. Hence, the first asexual reproduction that occurs in the host, schizogony in liver, is essential for increasing parasite numbers and establishing infection.

The metamorphosis of sporozoite into a replicative liver stage is associated with clearance of organelles, essential for sporozoite but dispensable after hepatocyte invasion, via secretory autophagy. We hypothesize that an unconventional mechanism analogous to exophagy may be used for organelle expulsion. We previously illustrated the significance of autophagy-related gene, PbATG8, localized to the membranes of apicoplast and cytosolic vesicles, in liver stage development through ATG8-overexpressing P. berghei. Through elevated expression of Plasmodium ATG8, we showed PbATG8 is essential for microneme compartmentalization before expulsion and apicoplast proliferation, and dysregulation of PbATG8 leads to poor development into late liver stage and production of immature and uninjective merozoites in small merosomes (Voss et al., 2016). Moreover, colocalization of PbATG8 with PbGRASP and PbVPS4, major players in the formation of amphisomes, suggests that these Plasmodium homologs may play a role in organelle expulsion through a UPS pathway during liver stage development.

We explored the role of GRASP in Plasmodium development by selectively deleting PbGRASP gene from P. berghei ANKA. From our analysis of EEF size, we suspect that PbGRASP-KO sporozoites suffer from mid-stage developmental defects as seen through somewhat smaller EEF size of PbGRASP-
KO, when compared to WT, at 22h p.i. (Figure 7, 8, 9, 10, 11, 12). The size of the parasite is positively correlated with its fitness and a smaller size may suggest a delay in early-mid schizogony development, characterized by DNA replication and selective organelle elongation. However, no other significant observed differences at any other time points, suggests that GRASP may not play a role in *Plasmodium* development beyond 22h p.i. Successful clearance of micronemes showed that GRASP is not essential for autophagy (Figure 10, 11, 12). Hence, we hypothesized that GRASP may act as a regulator for autophagy and gene deletion would lead to faster microneme clearance in KO. However, there were no observable differences in the quantification of TRAP signal inside the parasite and the PV lumen (Figure 13), suggesting that GRASP may not play a role in microneme clearance in contrast to our initial hypothesis. Although at 22h p.i., the PbGRASP-KO was smaller than WT, normalization of TRAP signal did not lead to any significant differences, suggesting that parasite size may not influence micronemal elimination. Alternatively, *Plasmodium* could have recruited other proteins to counter the lack of GRASP. However, to better understand the colocalization of GRASP seen with VPS4 and ATG8, we wanted to see if GRASP associates with other autophagy proteins and micronemes during the liver stage development, to see if localization of GRASP intersects with secretory autophagy.

The golgi protein, GRASP, is fairly conserved across species with a commercial antibody available based on the Rat-GRASP. However, the target sequence used for developing the antibody has no similarities with *P. berghei*. Hence, we planned to generate a PbGRASP specific antibody. We generated a recombinant plasmid expressing a synthetic gene consisting of immunogenic epitopes from PbGRASP with a drug cassette and a 6xHis tag (Figure 14, 15). Similar levels of gene expression were seen in both M15 and SG13009 chemically competent *E. coli* cells, containing pREP4 plasmid, suggesting that either cell strain could be used for gene expression. We chose to use M15 *E. coli* cell strain for further expression since they permit high-level expression and are easy to handle. On optimization, the expression levels of synthetic PbGRASP protein were found to be directly dependent on temperature while solubility reduced (Figure 16). The reduction in protein solubility was strongly
countered by the increase in expression levels, resulting in higher amounts of protein being synthesized at 37°C.

The high solubility of the target protein containing the 6x His tag meant that the protein can be purified in its native form using Ni-NTA affinity chromatography. However, when we purified the protein using this process, we see bands of multiple sizes suggestive of polymerization of target protein or aggregation (Figure 17). The non-specific binding of unrelated and nontagged proteins to the Ni-NTA resin is likely seen due to the purification being done in native conditions. We then purified the target protein using FPLC for reduced non-specific binding (Figure 18). However, due to the presence of urea, a reducing agent, we exchanged the buffer to PBS (pH=7.4), which has similar composition and ion concentration as human body (Figure 19). After producing the target synthetic protein in three different ways, synGRASP, synGRASP-U and synGRASP-P were used to immunize mice as described in Figure 5.

There were no significant differences observed between the antibody detection of anti-synGRASP antibody, anti-synGRASP-U antibody and anti-synGRASP-P antibody against dilutions of synGRASP-P, due to its usage in the booster dose (Figure 20). However, the difference of a single band in anti-His antibody blot vs multiple bands in synthetic PbGRASP-specific antibody blots meant there were proteins other than the target protein in synGRASP-P solution. We theorized that some unrelated proteins were eluted during FPLC purification but went undetected during Coomassie staining due to their relatively low amount, which may have led to non-specific antibodies. Nonetheless, this problem could be resolved by purifying the antibodies using antigen-specific affinity binding to obtain PbGRASP specific antibodies. However, unlike our initial results, the antibodies generated detect proteins of similar sizes at varying intensities between the WT and PbGRASP-KO (Figure 21). Although the size of these proteins coincides with missing proteins from PbGRASP-KO, when compared to WT, the antibody detection in KO lysate at a reduced amount suggests that the antibodies generated do detect PbGRASP, along with other proteins that share epitopes with purified unrelated proteins in the synGRASP-P.
Alternatively, the antibodies were generated against conformational epitopes of synthetic GRASP, different from epitopes of PbGRASP resulting in detection of proteins other than PbGRASP.

From IFA, we see that there is a lot of non-specific detection using anti-GRASP-P antibody in both WT and PbGRASP-KO (Figure 22). Moreover, in WT IFA, we do not see any clear and distinct puncta of PbGRASP distributed along the cytoplasm as should be seen in *P. berghei* (Kaiser et al., 2016). Hence, the alternative theory that these antibodies we generated do not, in fact detect PbGRASP, seems more likely and supports our observations. Since, our proposed PbGRASP-specific antibody generation strategy was not successful, we propose tagging the protein with small antibody or fluorescent tags in *P. berghei* ANKA genome for fluorescence imaging. Using the tagged *P. berghei* strain, we can study how the association of GRASP with autophagy machinery and other organelles likely to be involved in organelle expulsion varies as *Plasmodium* develops in liver. If the tag along PbGRASP can be expressed across *Plasmodium* stages, we can further use it to study golgi distribution during *P. berghei* development.

Another player we hypothesize has a role in *Plasmodium* development, especially in micronemal clearance is VPS4. We have previously seen continued expression of PbVPS4 network observed from 6h p.i. till 48h p.i., suggestive of its involvement in later exoerythrocytic development. Hence, to characterize the function of VPS4 during *Plasmodium* development, we are trying to obtain clonal populations of lab-engineered PbVPS4-KO. Initial cloning strategy only resulted in 4 mice getting infected out 50 mice, a mere 8% success rate, with 2 potential PbVPS4-KO clones (Figure 23). However, sequencing these clones has proved to be difficult due to either issues with PCR amplification or issues with purifying in the Genome Core Facility. We hypothesize that issues with PCR amplification could be due to contamination of gDNA or annealing temperature of primers. We can run a gradient PCR to analyze or eliminate if the primer annealing is the root of the problem. For addressing gDNA contamination or purification issues during sequencing, we can use the simple and easy-to-use gel purification technique to enhance purity levels.
Using the improved cloning protocol, we saw better results with a 50% infection rate and 3 potential PbVPS4-KO clones (Figure 24). However, sequencing reveals that we have failed to obtain pure clones of PbVPS4-KO this time (Figure 25), suggesting we rather obtained a mixed population due to infecting 2 parasites per mice. We can repeat the cloning step of PbVPS4-KO by infecting the mice with only 0.5-1 parasites to ensure we obtain clonal population of WT or PbVPS4-KO. Alternatively, it could also be possible to have obtained poorly engineered clones with the PbVPS4 gene still intact with a drug cassette also attached to the *P. berghei* genome. To test our hypothesis of poor PbVPS4-KO construct, we further extend the sequencing along PbVPS4-5’UTR to see if it leads to the drug cassette. If the construct needs to be re-engineered, we can design and transfected a strain with truncated VPS4 or a functional mutant that has either lost its function or overexpresses the gene.

After obtaining a clonal population, we can assess the significance of PbVPS4 gene by taking the cloned parasite through all the stages of *Plasmodium*. We will first infect mice with infected blood to phenotype mutation during blood stage. On feeding the infected mice to mosquitoes, we will analyze the oocyst development and sporozoite yield in salivary gland of mosquitoes. Next, the sporozoites will be used to study sporozoite gliding ability and phenotype VPS4 during liver-stage development, in vitro and in vivo, especially during micronemal expulsion. Furthermore, to assess our prediction of VPS4 role in schizogony development, we will continue to examine the development of *Plasmodium* into blood stage in vivo.
Figure Legend

Figure 1

(Note: Adapted Figure 1 from “Sexual development in Plasmodium parasites: knowing when it’s time to commit”, by G. A. Josling & M. Llinás, 2015, *Nature Reviews Microbiology*, 13(9), 573–587.)
Figure 1. The *Plasmodium* Life Cycle

The Plasmodium cycles between a definitive female mosquito host and a vertebrate intermediate host.

**Vertebrate host:** During a blood meal, the sporozoites are transmitted into the host dermis by an infected female *Anopheles* spp. mosquito vector as it injects saliva. The sporozoites invade and travel through the blood capillaries to access the liver and infect hepatocytes inside a parasitophorous vacuole. Post invasion, the slender sporozoites initiate schizogony and develop into a rounded metabolically active trophozoite. The trophozoite transforms into a hepatic schizont which bursts out of hepatocytes as meroosomes containing tens of thousands of merozoites. The hepatic merozoites are released into the blood to invade red blood cells (RBCs) and initiate the asexual reproduction cycle. The merozoites first convert into the ring-stage followed by erythrocytic trophozoite and schizont forms. The schizonts on lysing RBCs produce erythrocytic merozoites that continue the asexual cycle by invading new RBCs with very few schizonts committing to sexual development. The committed schizonts develop into transmission-competent sexual stage, gametocytes, on erythrocyte reinvasion which are then picked up by a mosquito during its next feeding.

**Mosquito vector:** During a blood meal, the female mosquito vector ingests both male and female gametocytes from an infected vertebrate host. The mature gametocytes initiate the sexual reproduction cycle by differentiating into gametes. Inside the midgut lumen, the male and female gametes fuse to create a zygote which metamorphoses into a motile ookinete. The ookinetes travels to the basal surface of midgut epithelium to convert into an oocyst. Within the oocyst, the asexual sporogony generate the sporozoite form of the *Plasmodium*. The sporozoites burst from the oocyst and migrate to the salivary gland tissues. Once inside the salivary gland, the sporozoites undergo final developmental changes and wait for the mosquito to take the next blood meal to continue the transmission cycle.
Figure 2

A. Progressive metamorphosis

B. TRAP, 10 nm gold particles

C. TRAP

D. PbATG8, ACP

E. PbATG8, 10 nm gold particles

F. Diagram showing apicoplast and Golgi vesicle

G. PbATG8, PbGRASP, PbVPS4
Figure 2. Metamorphosis of sporozoites and microneme elimination by autophagy

(A) Scanning EM on converting *P. berghei* maintained axenically. Bars, 1 µm. (B) Transmission EM of intrahepatic *Plasmodium yoelii*. Bars, 1 µm. (C) Double IFA using anti-TRAP and PbATG8 antibodies on intrahepatic *P. berghei* 12 h p.i., showing colocalization. (D) Double IFA using anti-PbATG8 and ACP antibodies on intrahepatic *P. berghei* 22 h p.i., showing colocalization. (E) ImmunoEM on intrahepatic *P. berghei* using anti-PbATG8 antibody, showing gold particles on the outermost membranes of the apicoplast (api, arrows). Bars, 100 nm [adapted from 9,13,17]. (F) Hypothetical model for microneme exophagy and amphisome detection (see text for description). (G) Triple IFA using anti-ATG8, anti-GRASP and anti-VPS4 antibodies on intrahepatic *P. berghei* 22 h p.i., showing significant co-association of ATG8, GRASP and VPS4 on same structures.
**Figure 3. Origin of synGRASP sequence**

The 273 bp long synGRASP gene was created by stitching together immunogenic epitopes (highlighted in yellow) of PbGRASP with triple glycine sequences (in green) between the restriction sites (in red) of BamHI and HindIII. The immunogenic epitopes were selected using the AbDesinger software. The synGRASP gene was then generated in a pDEF plasmid.
Figure 4

A

B

C

D

VECTOR

INSERT

RECOMBINANT PLASMID

53
Figure 4. Generation of pQE30-synGRASP recombinant plasmid

(A) The pQE30 plasmid as the vector showcasing the features including ATG (start codon), 6xHis Tag, AmpR (ampicillin resistance) and the restriction enzymes BamHI and HindIII. (B) The synGRASP gene as the insert with the restriction enzymes highlighted in yellow. (C) The BamHI/HindIII digested vector and insert, with the hanging sequences are ligated, to form the recombinant plasmid. (D) The recombinant plasmid pQE30-synGRASP with the synGRASP gene between the enzymes Bam HI and HindIII.
Figure 5

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Figure 5. Immunization strategy to generate PbGRASP specific antibodies

Seven days prior to immunization, pre-immune serum was collected from 6 BALB/c mice through retro-orbital bleeding. On the day of first dose of immunization, three different strategies were applied where 2-4 mice were intraperitoneally injected with 20-40 µg of one of the three proteins synGRASP, synGRASP-U or synGRASP-P, mixed with the Freund’s complete adjuvant. All mice were boosted with an intraperitoneal second dose 14 days later with 20-40 µg synGRASP-P mixed in Freund’s incomplete adjuvant.
Figure 6

The PbVPS4-KO was constructed with the help of E. coli cloning vector pDEF containing the human dihydrofolate reductase (hDHFR) drug selection marker for the mutant parasites. The PbVPS4 gene was replaced from the Plasmodium berghei genome through homologous recombination with the hDHFR cassette. Homologous arms, the 5’ UTR and the 3’ UTR, in the gDNA of P. berghei ANKA were amplified using PCR and inserted upstream and downstream of the drug selection cassette, respectively, via Gibson Assembly. Primers 1 and 2, flanking the 5’ UTR, and primers 3 and 4, flanking the 3’ UTR, are designed to confirm the presence of the hDHFR cassette in the cloned parasites.
Figure 7
Figure 7. Liver stage infection with PbGRASP-KO in cultured hepatocytes

(A) PV enumeration of exoerythrocytic forms (EEF) *in vitro*. Hepa1-6 cells were infected with WT or PbGRASP-KO parasites (clone 4) for 24h, 48h and 67h before fixation for IFA using anti-HSP70 antibody to count PV per 50 fields at each time point per coverslip. Data means ± S.D. of experiments done in triplicate. (B) Measurement of PV shape of EEF in vitro. Same infection protocol and IFA for HSP70 described in (A). Velocity software deconvolved images tagged with HSP70 fluorescence to obtain the average volumic area (volume in µm³) of WT and PbGRASP-KO PV at 4h, 10h, 22h and 36h p.i. Data are means ± S.D. No significant difference was observed between WT and PbGRASP-KO. (C-E) Measurement of PV volume of EEF in vitro. Same infection protocol and IFA for HSP70 described in (A). Velocity software deconvolved images tagged with HSP70 fluorescence to obtain the average volumic area (volume in µm³) of WT and PbGRASP-KO PV (n=35) at 4h, 10h, 22h, 36h, 48h and 67h p.i. Data are means ± S.D. Only at 22h p.i., the volume of PV from the PbGRASP-KO was statistically significantly smaller than PV from WT (p<0.0001, Ordinary one-way ANOVA with Tukey’s multiple comparisons test using GraphPad Prism)
Figure 8. Comparison of the distribution of TRAP-containing micronemes in WT and PbGRASP-KO liver forms at 4h p.i.

Triple IFA on WT and PbGRASP-KO liver forms 4h p.i. for immunostaining with anti-PbTRAP, anti-PbUIS4 and anti-HSP70 antibodies, showing punctates for TRAP 4h p.i. Representative images of parasites are shown.
Figure 9
Figure 9. Comparison of the distribution of TRAP-containing micronemes in WT and PbGRASP-KO liver forms 10h p.i.

Triple IFA on WT and PbGRASP-KO liver forms 10h p.i. for immunostaining with anti-PbTRAP, anti-PbUIS4 and anti-HSP70 antibodies, showing converting parasites, formation of the TVN (arrowheads) and TRAP puncta in the perikaryon area. Representative images of parasites are shown.
Figure 10
Figure 10. Comparison of the distribution of TRAP-containing micronemes in WT and PbGRASP-KO liver forms 22h p.i.

Triple IFA on WT and PbGRASP-KO liver forms 22h p.i. for immunostaining with anti-PbTRAP, anti-PbUIS4 and anti-HSP70 antibodies, showing smaller PV for the KO, expansion of TVN structures (arrowheads) and large and abundant TRAP puncta in the cytosol. Representative images of parasites are shown.
Figure 11

WT (36hpi)

(a) UIS4  (b) mHSP70  (c) TRAP
Figure 11. Distribution of TRAP-containing micronemes in WT liver forms 36h p.i.

Triple IFA on WT liver forms 36h p.i. for immunostaining with anti-PbTRAP, anti-PbUIS4 and anti-HSP70 antibodies, showing enlarged PV and progressive elimination of TRAP-micronemes (black arrow on the right, from panels a to d), with TRAP signal visible in the PV lumen (arrows) and within TVN structures (arrowheads). Representative images of parasites are shown.
Figure 12
Figure 12. Distribution of TRAP-containing micronemes in PbGRASP-KO liver forms 36 h p.i.

Triple IFA on PbGRASP-KO (clone 4) liver forms 36 h p.i. for immunostaining with anti-PbTRAP, anti-PbUIS4 and anti-HSP70 antibodies, showing enlarged PV and progressive elimination of TRAP-micronemes (black arrow on the right, from panels a to e), with TRAP signal visible in the PV lumen (arrows) and within TVN structures (arrowheads). Representative images of parasites are shown.
Figure 13

A: TRAP signal inside the parasite (in μm²)

B: Volume of TRAP inside the parasite normalized to HSP70 volume (μm²)

C: TRAP signal in the PV lumen (in μm²)

D: Volume of TRAP in the PV lumen normalized to HSP70 volume (μm²)
Figure 13. Quantification of TRAP signal in converting PbGRASP-KO in liver cells

(A-D) Data collected from WT and PbGRASP-KO parasites infecting liver cells 10h, 22h and 36h p.i. with representative liver forms shown in Figures 6-9. A and C show values of total volume for the TRAP signal that is detected within the parasite’s cytosol identifiable by HSP70 immunostaining. B and D are values in A and C normalized by the volume occupied by HSP70. Horizontal bars represent means from in dot plots.
Figure 14

A

BamHI/HindIII 4.0
3.4 kb
3.0
2.0

B

BamHI/HindIII 273 bp
Figure 14. Cloning synGRASP gene in pQE30 plasmid

(A) Post restriction digestion with the enzymes BamHI and HindIII, the size of pQE30 vector and synGRASP gene insert was verified using 1% Agarose gel to be 3.4 kb and 267 bp respectively. L1: 1 kb DNA ladder, L2: log 2 DNA ladder, UD V: undigested pQE30 vector, D V: digested vector, UD I: undigested insert (synGRASP gene in pDEF), D I: digested pDEF plasmid with synGRASP gene, D I (purification): For gel purification of synGRASP gene. (B) After transformation of recombinant plasmid pQE30-synGRASP in TOP10 E. coli cells, plasmid minipreps and restriction digestion with BamHI/HindIII were used to identify positive clones containing the 267 bp synGRASP gene insert. L1: log 2 DNA ladder, T1-T9: selected clones from pQE30-synGRASP transformed TOP10 cells, C: clone from vector-only (digested pQE30) transformed TOP10 cells as negative control. (C) Multiple sequence alignment of the sequence obtained from clone 9 (T9) with the template synGRASP gene using Clustal Omega v2.1. The start codon for translation and the 6x His Tag have been highlighted in yellow and red respectively.
Figure 15

A

BamHI/HindIII 273 bp

273 bp 0.2

B

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synGRASP
Figure 15. Expression of synGRASP gene in transformed SG13009 and M15 E. coli cells

(A) The clones of pQE30-synGRASP transformed SG13009 and M15 E. coli cells were screened using plasmid minipreps and restriction digestion to identify presence of synGRASP gene in all clones. L: log 2 DNA ladder, S1-S5: selected clones from pQE30-synGRASP transformed SG13009 cells, SC: clone from vector-only (digested pQE30) transformed SG13009 cells as negative control, M1-M5: selected clones from pQE30-synGRASP transformed M15 cells, MC: clone from vector-only (digested pQE30) transformed M15 cells as negative control. (B-C) Post inducing gene expression with IPTG, the gene expression in pQE30-synGRASP transformed SG13009 and M15 E. coli cells was checked using (B) Coomassie staining and (C) western blot with anti-His antibody, with ATG8-cko transformed M15 cells as positive control and pQE30 transformed SG13009 and M15 cells as negative control. L: 10-250 kDa protein ladder.
Figure 16

A

B
Figure 16. The synGRASP gene is optimally expressed at 37C in soluble fractions

(A-B) The gene expression in pQE30-synGRASP transformed M15 *E. coli* cells was optimized for temperature (25C, 30C, 37C) and solubility and checked using (A) Coomassie staining and (B) western blot with anti-His antibody. L: 10-250 kDa protein ladder, NI: non-induced control, I: induced control, S: soluble fractions, NS: insoluble fractions.
Figure 17

A

B

synGRASP
Figure 17. The synGRASP purified through Ni-NTA purification
The target protein was purified through Ni-NTA binding and eluted with buffers containing imidazole. (A-B) The flow through, washes and eluates collected during the purification were analyzed for the target protein using (A) Coomassie staining and (B) western blot with anti-His antibody. L: 10-250 kDa protein ladder, NI: non-induced control, IC: induced control, FT: flow through, W1-W3: washes collected using wash buffer, E1-E6: eluates collected during Ni-NTA purification. (C) The protein synGRASP, formed by combining eluates E4-E6, was quantified using a BCA assay. The OD values of the BSA standards from the plate reader were used to plot the standard curve. The concentrations of the sample (B5) and its sixth (A6) and eleventh (B6) dilutions were back calculated from the OD values using the cubic standard curve.
Figure 18

A

B
Plate 1

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</tbody>
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Standard Curve

Curve Fit: Cubic \( y = A + Bx + Cx^2 + Dx^3 \)

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Figure 18. The synGRASP-U purified using Fast Protein Liquid Chromatography

The purification of target protein was optimized through FPLC, done with the help of Dr. Abhishek Gupta, and eluated with buffers containing imidazole and urea. (A-B) Some eluates collected during the purification were analyzed for the target protein using (A) Coomassie staining and (B) western blot with anti-His antibody. L: 10-250 kDa protein ladder, NI: non-induced control, IC: induced control, E15-E19: eluates collected using FPLC purification. (C) The protein synGRASP-U, formed by combining eluates E16-E18, was quantified using a BCA assay. The cubic standard curve plotted from the OD values of BSA standards was used to estimate the protein concentration in the sample (A6) and its fifth (A7) and tenth (B7) dilutions.
**Figure 19**

### A

**Input sequence:**

```plaintext
```

Change at pH 5.6 (lysosome): 5.6  
Change at pH 7.4 (cytoplasm): 3.4  
Change at pH 8.8 (mitochondria): 9.9

Your protein (peptide) has 87 amino acids.

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<th>Count</th>
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</table>

Protein mass: 9540.52374 Da

### B

**Isoelectric point vs. molecular weight for all proteins**

- **Isoelectric point** – 4.553 (IPC2.protein.svr19)
- **Molecular weight** – 9.14 kDa
Isoelectric point of the protein is: 4.55

- average pl 4.36
- IPC2.protein.svr19 4.55
- IPC2_protein 4.55
- IPC_protein 4.42
- IPC2.peptide.Conv2D 4.67
- IPC2.peptide.svr19 4.47
- IPC2_peptide 4.52
- IPC_peptide 4.36
- Toseland 4.26
- Thurk11 4.27
- Nozaki_Tan 4.48
- Dawson 4.36
- DTASel ect 4.62
- EMBOS S 4.26
- Grimsley 4.17
- Patrickios 3.82
- Rodwell 4.25
- Sillero 4.53
- Solomon 4.36
- Lehninger 4.31
- Wikipedia 4.24
- ProMoST 4.66

* average pl includes all scales except Patrickios (highly simplified scale frequently leading to bizarre results)
D

### Table 1: Plate 1

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<tr>
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<td>-0.057</td>
<td>-0.057</td>
<td>-0.057</td>
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</tbody>
</table>

### Standard Curve

**Formula:**

\[ y = A + Bx + Cx^2 + Dx^3 \]

**Curve Fit Results**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Estimated Value</th>
<th>Std. Error</th>
<th>Confidence Interval</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>-0.021</td>
<td>0.019</td>
<td>[-0.075, 0.033]</td>
</tr>
<tr>
<td>B</td>
<td>2.080</td>
<td>0.104</td>
<td>[1.790, 2.370]</td>
</tr>
<tr>
<td>C</td>
<td>-0.817</td>
<td>0.132</td>
<td>[-1.185, -0.450]</td>
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<td>D</td>
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### Sample Table

<table>
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<th>CV</th>
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<tr>
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<td>0.732</td>
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<td>0.426</td>
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<td>0.0</td>
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</table>
Figure 19. Predicted isoelectric point of synthetic PbGRASP in silico to be 4.55 using Isoelectric Point Calculator 2.0

The stitched sequence of selected epitopes from PbGRASP were input into the online tool, Isoelectric Point Calculator 2.0 (IPC 2.0), to computationally predict the isoelectric point. IPC 2.0 is a web server developed to predict pKa dissociation constant and isoelectric point based on sequence information using machine learning algorithms and support vector regression for ensemble of 9 Multi-Layer Perceptron models. (A) IPC 2.0 calculates the number of amino acids, molecular weight and the charge at different pH for the input sequence. (B) Plot of isoelectric point vs molecular weight of the input sequence. (C) Isoelectric point of the input sequence predicted using various algorithms. For proteins (>60 aa), the prediction is based on the recommended methods - IPC2.protein.svr.19, IPC2_protein and IPC_protein. (D) The protein concentration of the protein, synGRASP-P, in PBS buffer was estimated from plotting the OD value measured in the B5 well with respect to the cubic standard curve.
Figure 20
Figure 20. The antibodies raised against synthetic PbGRASP in BALB/c mice using various techniques detect synGRASP-P

(A-E) The detection activity of anti-synGRASP antibody, anti-synGRASP-U antibody and anti-synGRASP-P antibodies against synGRASP-P was tested using western blots with primary antibodies (A) pre-immune sera as negative control, (B) anti-His antibody as positive control, (C) anti-synGRASP antibody, (D) anti-synGRASP-U antibody and (E) anti-synGRASP-P antibody. L: 10-250 kDa protein ladder, PbE: *Plasmodium berghei* extract.
Figure 21
Figure 21. The anti-synGRASP-P antibody detects WT *P. berghei* and potentially PbGRASP-KO

(A-B) Western blots were run to check antibody detection against WT *Plasmodium berghei* vs PbGRASP-KO with primary antibodies (A) anti-blood stage *P. berghei* IgG antibody as positive control and (B) anti-synGRASP-P antibody. L: 20-220 kDa protein ladder, WT: wild type *P. berghei*, KO: PbGRASP-KO. (C) Western blot to monitor if anti-synGRASP-P antibody shows any detection against increasing volumes of PbGRASP-KO. L: 20-220 kDa protein ladder, WT: wild type *P. berghei* as positive control, KO: PbGRASP-KO, 10-30: volume of parasite lysate-sample buffer mixture in microliters.
Figure 22. Immunofluorescence assay using anti-GRASP-P antibody

A PbGRASP-KO and WT infection of Hepa1-6 cells fixed at 24h p.i. observed by fluorescence microscopy. *Plasmodium* PV (PbUIS4) and nucleus (DAPI) were used to show detection ability of anti-GRASP-P antibody against PbGRASP. All bars, 5 microns.
Figure 23

Figure 23. PbVPS4-KO diagnostic PCR for mice using old protocol

Fifty Swiss Webster mice were used to clone the PbVPS4-KO using limiting dilution, injecting 1 parasite per mouse. Using the old protocol as mentioned above, only 4 mice (M1-M4) were identified to be infected using blood smears. (A-B) PCR for primer pairs (A) 1/2 and (B) 3/4 were set up to amplify the 2240 bp of 5’UTR and 1360 bp of 3’ UTR, respectively. The clones that show amplified bands for both regions are identified to be potential PbVPS4-KO clones. L: log 2 DNA ladder.
Figure 24
Figure 24. PbVPS4-KO diagnostic PCR for mice using new protocol

Ten Swiss Webster mice were infected with two parasites per each mouse to clone the PbVPS4-KO using the new protocol for limiting dilution, as mentioned in text above. Blood smears identified 5 out of 10 mice (C1-C5) to be infected. (A) PCR for primer pairs 1/2 and 3/4 were set up to amplify the 2240 bp of 5’UTR and 1360 bp of 3’ UTR, respectively. (B) A PCR with gradient for annealing temperatures identified higher temperatures to be ideal for primers 3 and 4. (C) With an annealing temperature of 55°C, a PCR was set up for potential clones identified previously for reverification. L: log 2 DNA ladder.
Figure 25

A

B

C
Figure 25. Sequence alignment of potential PbVPS4-KO clones

Potential PbVPS4-KO clones identified from PCR were selected for PCR amplification of the segment between the primers 1 and 4, and subsequently sent for sequencing with the primers 1-4 and a VPS4_Rev primer, designed specifically to verify presence of PbVPS4 gene. (A-C) Sequence alignments of clones (A) C2, (B) C3 and (C) C4 with *Plasmodium* gene segment containing the 5'UTR, PbVPS4 gene and 3'UTR regions. (D) Alignment of sequences obtained from C2-C4 clones using the VPS4_Rev primer with PbVPS4 gene as a reference.
Table 1

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<th>Protein</th>
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<th>Comments</th>
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<td>synGRASP</td>
<td>Elution buffer</td>
<td>Mixed eluates E4-E6 from Ni-NTA purification</td>
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<td>synGRASP-U</td>
<td>Protein Elution Buffer</td>
<td>Mixed eluates E16-E18 from FPLC purification</td>
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<tr>
<td>synGRASP-P</td>
<td>PBS</td>
<td>Exchanged buffer of synGRASP-U to PBS (pH = 7.4)</td>
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Table 1. Proteins quantified using Pierce™ BCA Protein Assay

Three different proteins synGRASP, synGRASP-U and synGRASP-P were created from the proteins purified using various approaches, as described in the comments. These proteins were quantified using Pierce™ BCA Protein Assay with the bovine serum albumin standards in respective diluents.
Table 2

<table>
<thead>
<tr>
<th>Primary Antibody</th>
<th>Target</th>
<th>Secondary Antibody</th>
<th>Alexa Fluor</th>
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<tr>
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<td>goat anti-mouse</td>
<td>Alexa-488</td>
</tr>
<tr>
<td>mouse anti-synGRASP-P</td>
<td>GRASP</td>
<td>goat anti-mouse</td>
<td>Alexa-488</td>
</tr>
<tr>
<td>rabbit anti-TRAP</td>
<td>TRAP micronemes</td>
<td>goat anti-rabbit</td>
<td>Alexa-647</td>
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<tr>
<td>goat anti-UIS4</td>
<td>Parasitophorous vacuole</td>
<td>donkey anti-goat</td>
<td>Alexa-568</td>
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</table>

Table 2. The different primary and secondary antibodies with respective fluorescence tags used for immunofluorescence assays

To study the morphological differences between PbGRASP-KO and WT *P. berghei*, the primary antibodies mouse anti-HSP70 (4C9), rabbit anti-TRAP and goat anti-UIS4 with their respective secondary antibodies and fluorescence tags were used.

For testing the detection activity of anti-synGRASP-P raised in mouse, anti-UIS4 antibody was also used to identify the parasite through its PV during IFA.
Table 3

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<td>VPS4-Rev.</td>
<td>5’-CAGTGTCTCCATATCCGTCA-3’</td>
<td>53°C</td>
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</table>

Table 3. Primers used for PbVPS4-KO verification

In the PbVPS4-KO, the primers 1 and 2 amplify 2240 bp region overlapping the 5’ UTR and the hDHFR cassette while primers 3 and 4 amplify 1360 bp region overlapping the hDHFR cassette and the 3’ UTR.
References


