Spring 4-25-2014

An Exported Malaria Protein Regulates Glucose Uptake During Intraerythrocytic Infection

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By

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A dissertation presented to the
Graduate School of Arts and Sciences
of Washington University in
partial fulfillment of the
requirements for the degree
of Doctor of Philosophy

May 2014

St. Louis, Missouri

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ACKNOWLEDGEMENTS

The completion of this dissertation was made possible by the support of several individuals and by financial support from Howard Hughes Medical Institute. I am thankful to my committee members for their guidance and encouragement over the years. I am grateful to my lab mates for helpful discussion, suggestions, ideas, and for providing a fun and educational lab environment. I would especially like to thank Natalie Spillman for her perseverance and for her help with a significant part of my thesis work. I thank Paul Sigala for help with revisions and being available for general questions and discussions. I thank Josh Beck for help with revisions, experiments, and discussions. I especially thank Anna Oksman for her support and her friendship. I am extremely grateful to my advisor, Daniel Goldberg, for taking me into the lab and guiding me through my graduate work. Thanks to Dan, I have confidence in myself, my thought process, and my ability to think independently as a scientist. I would also like to thank my undergraduate mentor, Dr. James H. Bouyer. Dr. Bouyer has been a father figure as well as a mentor. I am forever thankful for him and to him. I thank my parents for always telling me how proud they are of me and for their support. I am so blessed to have the support of best friends of eighteen years, Danielle Snipes, Akilah Swinton, and Stephanie Glover. I thank my family, especially my sister Quiana Gaither, for their endless support and encouragement. I thank my spouse, Talarrius Likely, for motivating me, supporting me, and loving me. So many people have encouraged and supported me throughout my life. I am grateful for and thankful to every one of them.

ABSTRACT OF THE DISSERTATION

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Malaria is the world's second biggest infectious killer after tuberculosis. It accounts for 219 million cases each year, with an estimated 660,000 deaths. The majority of these deaths occur in sub-Saharan Africa, in children under 5 years old. In addition to Africa, malaria is endemic to Asia, Central and South America, the Caribbean and the Middle East.

Plasmodium falciparum (P. falciparum) is the protozoan parasite that is responsible for the deadliest form of human malaria. *Plasmodia* are carried by the female Anopheles mosquito and infected into humans during a blood meal. The parasites invade liver cells and form merozoites which erupt from liver cells to invade red blood cells. The intraerythrocytic cycle of infection is responsible for the clinical manifestations of malaria, namely fever and chills. The intraerythrocytic cycle is also the stage of disease that is most studied and targeted for treatment.

Although treatment for malaria is available, drug-resistant forms of the parasite are increasingly rampant. For this reason, new, more effective treatments for malaria are necessary. To develop these treatments, we must have a better understanding of the biological processes that the parasite employs to survive in the host to cause disease.

In 1996, an international effort was launched to sequence the genome of *P. falciparum* with the expectation that the genome sequence could be exploited in the search for new drugs and vaccines to fight malaria. In 2002, the genome sequence was published with gaps in some chromosomes. Approximately 5,300 protein-encoding genes were identified; of these about 60% were labeled as hypothetical proteins.

Our studies focus on determining the function of one hypothetical protein, PFB0923c, that we now call Glucose Uptake Restoration Protein (GURP). We show that GURP localizes to novel double membrane vesicles in the RBC cytosol and is essential during *P. falciparum* intraerythrocytic infection. GURP interacts with and sequesters the host protein stomatin, which is known to depress glucose uptake in mammalian cells. Knockdown of GURP decreases glucose uptake and impairs parasite growth in RBCs. This phenotype can be rescued with antioxidants, suggesting that hexose monophosphate/pentose phosphate pathway impairment is lethal in the knockdown parasites. GURP C183 is essential to parasite viability and trafficking of GURP vesicles to the RBC cytosol. Together, these data demonstrate that GURP is essential to *P. falciparum* viability and glucose uptake during infection of red blood cells.

CHAPTER I

Introduction

APICOMPLEXA

The phylum Apicomplexa consists of a wide range of organisms that are largely parasitic (Figure 1). These unicellular eukaryotes are obligate intracellular parasites that take over the host cell after invasion. Apicomplexans are characterized by a number of organelles in the apical end of the organism, hence the name, involved in host cell attachment, invasion, and the establishment of an intracellular parasitophorous vacuole within the host cell (Morrison, 2009).

Apicomplexans are furtive invaders, sheltering from the immune response in the cells of their hosts, while at the same time using these cells as a source of nutrients and as a space for development (Striepen *et al.*, 2007). When the parasite has replicated and is ready for egress, the daughter parasites burst from the current cell to invade and occupy a new cell and begin the developmental process anew.

Members of the phylum include: *Toxoplasma gondii*, the causative agent of toxoplasmosis; *Cryptosporidium*, a waterborne pathogen with implications for immune-compromised persons; *Gregarina*, an invertebrate parasite; and *Plasmodium*, the etiological agent of malaria (Wasmuth *et al.*, 2009), which is the subject of this dissertation.

PLASMODIUM

Plasmodium was first seen by Charles Louis Alphonse Laveran in 1880 (Mundwiler-Pachlatko and Beck, 2013). In 1898, Giovanni Battista Grassi and Ronald Ross both showed that *Plasmodium* existed in the wall of the midgut and salivary glands of a *Culex* mosquito using bird species as the vertebrate host. Grassi showed that human malaria could only be transmitted by *Anopheles* mosquitoes.

There are several hundred species of *Plasmodium*, five of which cause malaria in humans: *P. vivax, P. malariae, P. ovale, P. falciparum* (Richie, 1988) and *P. knowlesi* (White, 2008). *P. falciparum and vivax* species are the most common cause of malaria (World Health Organization (WHO), 2014). *P. falciparum* is by far the deadliest species of human malaria. It causes severe infection that kills hundreds of thousands of people every year (WHO, 2014). Apicomplexans have multi-stage life cycles usually involving development within 2 hosts. *P. falciparum* infects both female *Anopheles* mosquitoes and humans (Figure 2).

Mosquito Midgut

The *Plasmodium* parasite undergoes sexual reproduction once during a life cycle, in the mosquito. Gametogenesis is temperature and pH dependent and occurs in the mosquito midgut lumen (Figure 2). Male gametocytes fertilize female gametocytes to form zygotes that undergo meiosis and genetic recombination to transform into an ookinete. The ookinete goes on to invade the midgut epithelium and migrate through the epithelial cells (Baton and Ranford-Cartwright, 2005). Ookinetes transform into oocysts at the end of their migration through the midgut epithelium. These oocysts develop into thousands of sporozoites which burst from the oocyst and invade the mosquito salivary glands (Aly *et al.*, 2009). When the mosquito takes a

blood meal, the sporozoites are injected into the human host. These sporozoites then breach the capillaries, enter the bloodstream and travel to the liver to invade hepatic cells (Young, *et al.*, 2005).

Liver Stage

After traversing through a number of hepatocytes, the sporozoite actively invades a final hepatocyte with the formation of the parasitophorous vacuole (PV) (Mota *et al.*, 2001). Within hepatic cells, sporozoites differentiate into exo-erythrocytic forms and undergo asexual reproduction that results in the formation of tens of thousands of merozoites (Vaughan *et al.*, 2008). Merozoites are packaged into specialized vesicles called merosomes which are released from hepatic cells and enter the bloodstream. These merozoites invade red blood cells, initiating the intraerythrocytic cycle (Prudencio *et al.*, 2006).

Intraerythrocytic Cycle

Invasion

Parasite merozoites adhere to the host red blood cell, first through low affinity interactions (Bannister and Dluzewski, 1990) followed by apical reorientation and high affinity binding of parasite erythrocyte binding-like (EBL) proteins and sialic acid on host glycoproteins (Bannister and Mitchell, 2003; Malpede *et al.*, 2013). A tight junction forms at the attachment site of the merozoite to the red cell membrane, creating an invagination in the eryrthrocyte membrane (Aikawa *et al.*, 1978; Keeley and Soldati, 2004). As the parasite pushes its way into the erythrocyte , it creates a parasitophorous vacuole (PV).

The lipids that make up the parasitophorous vacuole membrane (PVM) are largely derived from the plasma membrane of the red blood cell. Since the RBC lacks the machinery to

synthesize new lipids or proteins as the parasite grows, the PVM is expanded and modified by the parasite, providing a customized environment for development (Cesbron-Delauw *et al.*, 2008).

Developmental Stages

Ring

After successful merozoite invasion of the RBC, the parasite develops into the ring stage. Morphologically, the parasite resembles a biconcave disc with its nucleus appearing as a dot on the disc (Langreth, 1978; el-Shoura, 1994). Late ring stage parasites begin the process of exporting parasite proteins to modify the host RBC (Bannister and Mitchell, 2003). Ring stage parasites then develop into trophozoites. The process of hemoglobin digestion begins in the late ring stage (Bakar et al, 2010).

Trophozoite

Trophozoites are the most active stage of parasite development. New proteins are exported into the RBC, more hemoglobin is degraded (Gluzman *et al.*, 1994; Olliaro and Goldberg, 1995), and the parasites attain more mass at the trophozoite stage. New permeability pathways are established for uptake of nutrients/efflux of wastes (Ginsburg *et al.*, 1983) and the presence of hemozoin, from digestion of hemoglobin, is readily visible in the parasite food vacuole (Slater and Cerami, 1992).

Schizont/Merozoite

At the schizont stage, the parasite prepares itself to egress from its current RBC and invade a new RBC. About 16 nuclei are generated and packaged into individual merozoites. The

merozoites contain secretory vesicles necessary for RBC invasion. The merozoite surface is coated with proteins that facilitate adhesion to the RBC surface (Bannister and Mitchell, 2003). Individual merozoites are released via protease-mediated rupture of the PVM and the RBC membrane (Wickham *et al.*, 2003; Soni *et al.*, 2005; Gelhaus *et al.*, 2005). The cyclical rupture of RBCs gives rise to fever and chills, common clinical symptoms of malaria.

Gametocytes

Approximately seven to ten days after the initial asexual cycle, gametocytes are produced in detectable numbers (Day *et al.*, 1998; Eichner *et al.*, 2001). Early gametocyte stages are sequestered in the bone marrow and spleen (Farfour *et al.*, 2012; Tiburcio *et al.*, 2012), while late stage gametocytes are released into the blood circulation. After several days of circulation, mature gametocytes become infectious to mosquitoes (Smalley and Sinden, 1977; Lensen *et al.*, 1999). When a mosquito takes a blood meal from an infected person, gametocytes are also taken into the mosquito midgut and the cycle starts again.

MALARIA

Malaria-causing parasites infect approximately 219 million people each year, with an estimated 660,000 deaths, mostly in Africa (UNICEF, 2013). It is the leading cause of death and disease in many developing countries, where young children and pregnant women are the groups most affected. Direct costs have been estimated to be at least $12 billion per year (Centers for Disease Control and Prevention [CDC], 2012).

Symptoms of malaria typically include chills, followed by fever and sweating. Headache, fatigue, muscular pains, and nausea are also likely to occur. The symptoms first appear ten to sixteen days after the infectious mosquito bite (National Institutes of Health [NIH], 2009). All of the clinical symptoms of malaria are associated with the blood stage of the parasite life cycle (Schofield, 2007).

P. falciparum malaria results in severe complications that involve the nervous, respiratory, renal, and hematopoietic systems (Trampuz *et al.*, 2003). Cerebral malaria causes brain swelling that may lead to brain damage (Senanayake and Roman, 1992). Acute lung injury leads to fluid accumulation in the lungs, which causes difficulty breathing (Gachot *et al.*, 1995). Kidney and liver failure is common in *falciparum* malaria (Prakash *et al.*, 1996), as is severe anemia (Trampuz *et al.*, 2003).

Malaria-causing parasites have become widely resistant to a number of common anti-malarial drugs (Shanks, 2006). Thus, new drug treatments are sorely needed to combat the spread of this disease. During intraerythrocytic infection, *Plasmodium* remodels the host red blood cell to satisfy its requirements for survival. Essential to this remodeling is export of parasite proteins into the RBC and their targeting to the RBC membrane and parasite derived

organelles. A good portion of exported proteins generally do not have homologues (Sargeant *et al.*, 2006), therefore, we can target them without disruption of human proteins.

HOSTILE TAKEOVER: RESTRUCTURING THE HOST CELL FOR PARASITE SURVIVAL

The erythrocyte lacks the major histocompatibility complex and a nucleus, providing an immunologically privileged environment for the parasite to develop. However, the uniform composition of this niche also poses challenges, requiring extensive host cell modifications (Heiber *et al.*, 2013). *Plasmodium falciparum* induces extensive host cell remodeling mediated by the export of hundreds of parasite proteins into the erythrocyte and under and within the host cell membrane (Cowman and Goldberg, 2010). The renovation of host erythrocytes by exported parasite proteins ensures the parasite's survival and contributes to its virulence and pathogenesis.

Most parasite proteins are targeted for export by the presence of a *Plasmodium* export element (PEXEL) sequence (Marti *et al.*, 2004; Hiller *et al.*, 2004). The PEXEL is a pentameric amino acid sequence-RxLxE/Q/D, where x is any amino acid- located 20-30 amino acids C-terminal from the signal sequence in proteins destined for export. The signal sequence targets these proteins to the ER where they are cleaved by plasmepsin V (Boddey *et al.*, 2013; Russo *et al.*, 2010) and subsequently translocated through the putative export machinery (PTEX) (Bullen *et al.*, 2012) and finally trafficked to their final destination in the host cell (Figure 3).

Plasmodium spp. also express a class of exported proteins, called PEXEL-negative exported proteins (PNEPs), that do not contain a PEXEL motif. PNEPs cannot be cleaved by plasmepsin V; therefore, they may use a trafficking pathway in the ER distinct from that of PEXEL proteins (Boddey and Cowman, 2013). Parasite exported proteins play a significant role in restructuring the RBC to the benefit of *Plasmodium*. These changes include increasing RBC permeability, inducing adhesion to vascular epithelial cells to avoid clearance in the spleen, and generating organelles to direct protein trafficking (Marti and Speilmann, 2013).

Plasmodial Surface Anion Channel (PSAC)

Parasite growth in red blood cells imposes a major demand for supply of nutrients and disposal of waste products (Cabantchik, 1990) and thus increases the permeability of the RBC to a broad range of small solutes (Overman, 1948; Kirk *et al.*, 1994; Ginsburg *et al.*, 1985; Cabantchik, 1990; Upston *et al.*, 1995; Saliba *et al.*, 1998; Staines *et al.*, 2000). One hypothesis in the field attributes these permeability changes to the plasmodial surface anion channel (PSAC) (Desai *et al.*, 2000; Kokhari *et al.*, 2009; Desai *et al.*, 2004). The PSAC-associated protein, cytoadherence-linked asexual protein 3 (CLAG3), is exported from the parasite and localizes to the red blood cell membrane (Nguitragool *et al.*, 2011). CLAG3 function in nutrient uptake renders the PSAC essential to intracellular parasite survival.

Knobs

Knobs facilitate the adherence of infected red blood cells (iRBCs) to the vascular endothelium (Crabb *et al.*, 1997). This adherence is critical to prevent the circulation iRBC's containing mature parasites, whose enhanced rigidity compared to uninfected RBCs would otherwise subject them to splenic filtration and destruction (Leech *et al.*, 1984),. Knobs consist predominantly of the knob-associated histidine-rich protein (KAHRP), assembling on the cytoplasmic face of the membrane (Rug *et al.*, 2006). *Plasmodium falciparum* erythrocyte membrane protein 1 (PfEMP1), a variant surface antigen and adhesin, is also a part of the knob structure. PfEMP1 binds to several host receptors to mediate adherence of the iRBC (Rug *et al.*, 2006). PfEMP1 is trafficked to the erythrocyte surface through Maurer's clefts (Goldberg and Cowman, 2010), parasite-derived organelles involved with trafficking of exported parasite proteins.

Maurer's Clefts

Maurer's clefts (MCs) are flattened vesicular structures beneath the red blood cell membrane (Sam-Yellowe, *et al.*, 2004), first identified in 1902 by Georg Maurer. MCs are responsible for transporting parasite proteins, such as PfEMP1, to their final destination in the red blood cell. (Hinterberg *et al.*, 1994; Adisa *et al.*, 2001; Hayashi *et al.*, 2001; Wickham *et al.*, 2001; Haldar *et al.*, 2002; Przyborski *et al.*, 2003; Bhattacharjee *et al.*, 2008). Recently, Maurer's clefts have been shown to be implicated in merozoite release, which depends on the phosphorylation of a MC resident protein (Blisnick *et al.*, 2005).

J-dots

The recently discovered J-dots are so named because the known contents are Hsp40 J-proteins. These structures are highly mobile , are tightly associated with the red blood cell membrane, and are thought to be involved in trafficking of parasite-encoded proteins through the cytosol of the iRBC (Kulzer *et al.*, 2010, 2012).

P. falciparum contains a PEXEL-based exportome of 300-400 proteins (Hiller *et al.*, 2004; Marti *et al.*, 2004; Sargeant *et al.*, 2006; van OOij *et al.*, 2008). Of these, 25% are phylogenetically unrelated to any known proteins (Sargeant *et al.*, 2006). These "exported proteins of unknown function" or "hypothetical proteins" may be playing a critical role in pathogenesis during RBC infection, and thus, may provide attractive drug targets.

INTO THE ABYSS: HYPOTHETICAL PROTEINS OF *Plasmodium falciparum*

The *Plasmodium falciparum* genome was published in 2002 in an effort to better understand parasite biology and develop new drug treatments. The nuclear genome is composed of 22.9 megabases distributed among 14 chromosomes (Gardner *et al.*, 2002). Of the 5,268 predicted proteins encoded, about 60% did not have sufficient similarity to proteins in other organisms to justify provision of functional assignments (Gardner *et al.*, 2002). These proteins were subsequently annotated as "hypothetical proteins." Currently, almost 50% of the genome remains classified as such (Balu, 2012).

My thesis project was aimed at characterizing one of these hypothetical proteins in order to significantly contribute to the understanding of *P. falciparum* biology. To begin, we screened the Plasmodium genome database, PlasmoDB, for proteins that had the following features: 1) a molecular weight less than 30 kiloDaltons (kDa), 2) evidence for expression expressed during the intraerythrocytic cycle and 3) an odd number of cysteines. The rationale for these criteria are explained below.

1) The parasite genome is 80% A-T rich (Gardner *et al.*, 2002) which can often lead to difficulties amplifying the gene of interest. We chose proteins with a molecular weight less than 30 kDa to facilitate cloning the gene for further functional characterization, including recombinant protein expression and purification.

2) As aforementioned, the intraerythrocytic cycle is responsible for the manifestation of the clinical symptoms of malaria. This developmental stage is also amenable for in vitro culture and investigation. Therefore, our research focuses on this stage of parasite development. We

wanted to ensure that the chosen protein was expressed during the clinically relevant RBC stage such that its biological properties could be readily investigated.

3) Free cysteines are frequently observed in functionally important sites in proteins such as catalytic, regulatory, and cofactor binding sites (Marino and Gladyshev, 2010). The criterion of an odd number of cysteines was chosen to increase the likelihood of finding a protein with a functionally important free cysteine that could be used as a starting point for mutagenesis as a step towards determining protein function.

Using these parameters we obtained a list of 19 exported hypothetical parasite proteins. Here, I will tell you about the characterization of one of those proteins that we call Glucose Uptake Restoration Protein, or GURP. Based on experiments described herein, we believe that GURP acts to regulate the infected red cell's glucose uptake through interaction with the host protein stomatin.

SWEET TOOTH: GLUCOSE USAGE in *Plasmodium falciparum*

Blood-stage *Plasmodium* derives most of its energy requirements from the metabolism of glucose via glycolysis (Scheibel, 1988). It has been documented that the parasite-infected red blood cells utilize glucose at a rate much higher than that of uninfected red blood cells (Oelshlegel *et al.*, 1975; Roth *et al.*, 1982; Roth *et al.*, 1988; Mehta *et al.*, 2006). Without intracellular energy stores during most of their life cycle, parasites are dependent on a constant supply of glucose from the host red blood cell (Patel *et al.*, 2008). Glucose is converted to glucose-6-phosphate (G6P), which is used in both glycolysis and the pentose phosphate shunt (Figure 5), and glucose deprivation leads to decreased ATP levels and decreased parasite cytosolic pH (Saliba *et al.*, 2004).

Conversion of glucose to G6P is catalyzed by the glycolytic enzyme hexokinase. Vertebrates have four hexokinase (HK) isoforms (Gonzalez *et al.*, 1964; Katzen and Schimke, 1965; Grossbard and Schimke, 1966), HKI, II, III, and IV. In human red blood cells, two isozymes are present: HKI and hexokinase R (HK-R) (Murakami *et al.*, 1990). The HK-R nucleotide sequence is identical to HKI except for the 5' extreme end (Murakami and Piomelli, 1997). As the RBC matures, HKI replaces HK-R, thus mature erythrocytes contain only 2-3% of the HK activity of reticulocytes (Shinohara *et al.*, 1985). Parasitized RBCs demonstrate a 25-fold increase of overall HK activity compared with uninfected RBCs (Roth, 1987; Roth *et al.*, 1988). As *P. falciparum* HK (PfHK) has an approximately four-fold lower substrate affinity for glucose compared with human HK (Roth, 1987), it is likely that most of the increase in HK activity is due to the erythrocyte HK.

Glut1is the major glucose transporter in red blood cells (Mueckler *et al.* 1985). The major erythrocyte membrane protein, stomatin, has been shown to associate with Glut1 (Zhang *et al.*,

1999, 2001; Rubin *et al.*, 2003; Kumar *et al.*, 2004; Rungaldier *et al*., 2013) and to reduce Glut1 affinity for glucose (Zhang *et al.*, 2001). Glucose uptake (defined as glucose transport and phosphorylation (metabolism)) is drastically increased in *P. falciparum* iRBCs (Homewood and neame, 1974; Neame and Homewood, 1975; Sherman and Tanigoshi, 1974; Tripatara and Yuthavong, 1986; Izumo *et al.*, 1989; Tanabe, 1990; Kirk *et al.*, 1996), indicating that the parasite has overcome stomatin's repression of Glut1 glucose uptake. My thesis work on the parasite protein, GURP, has shed light on a potential mechanism for regulation of glucose uptake during intraerythrocytic infection.

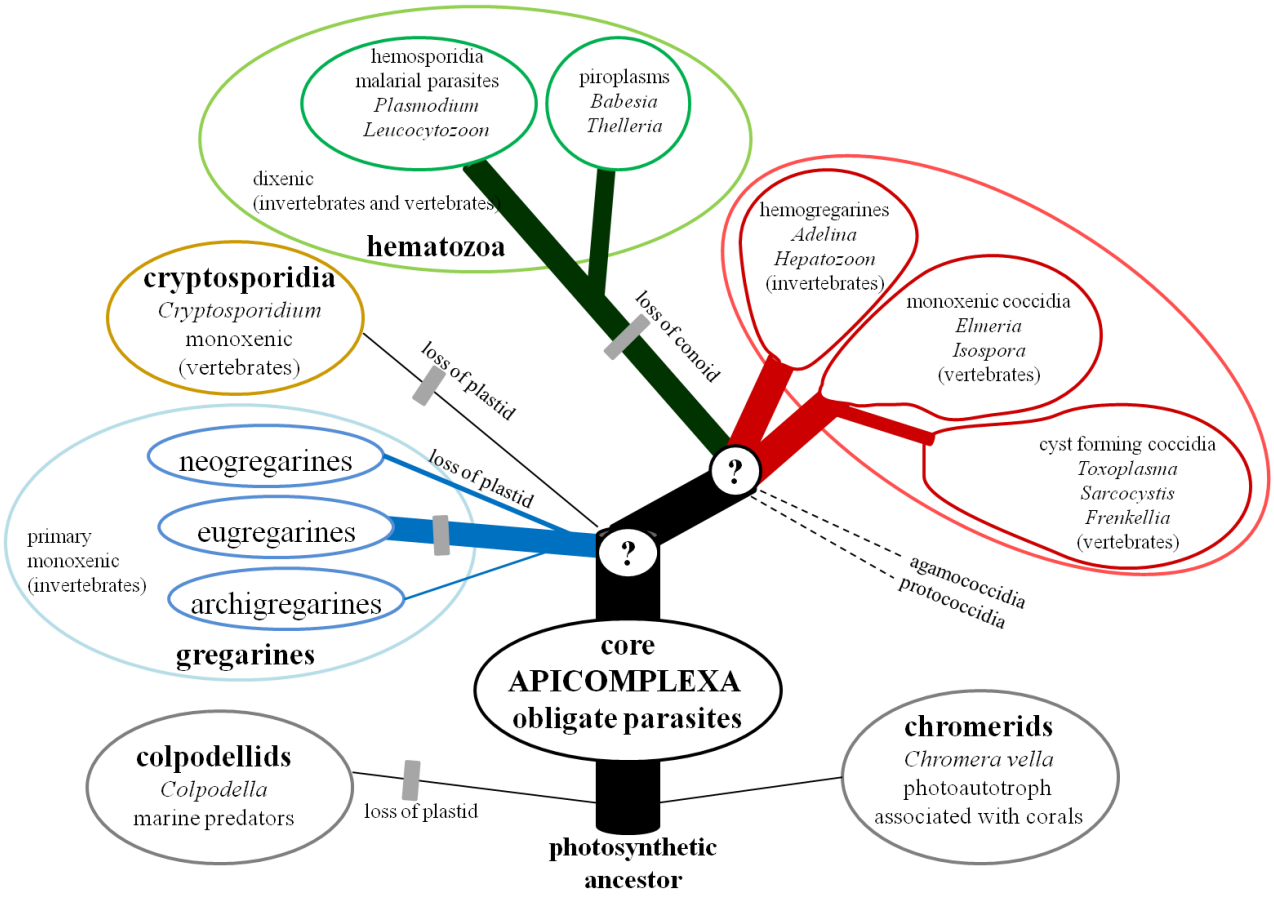

Figure 1. Apicomplexa phylogeny.

Tree showing the hypothetical phylogeny of the Apicomplexan phylum. Gregarina, Coccidia, and Hematozoa are the tree primary clades. Branch thickness indicates diversity. Adapted from (Slapeta and Morin-Adeline, 2011).

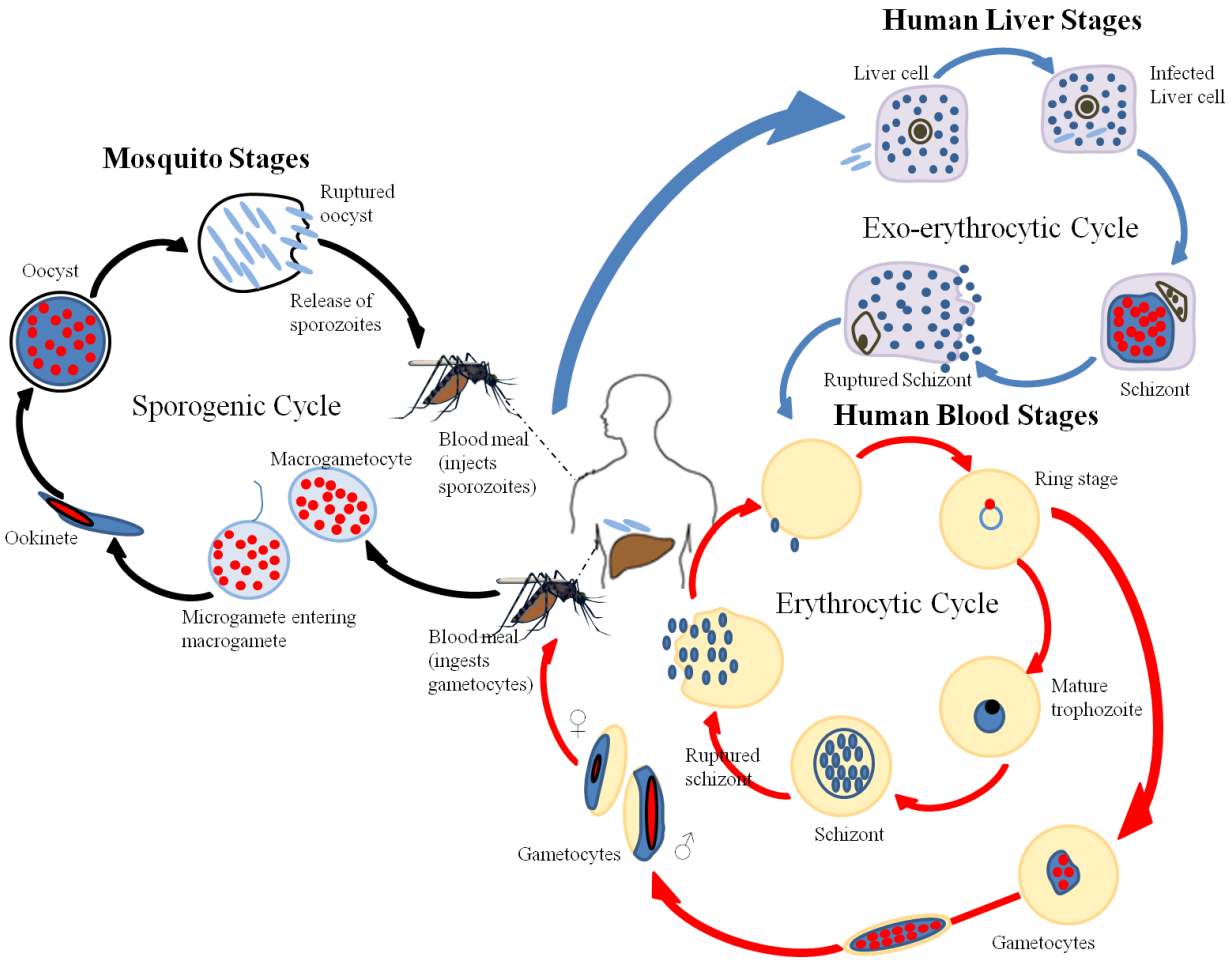

Figure 2. *Plasmodium* life cycle.

The *Plasmodium* life cycle involves two hosts: the *Anopheles* mosquito and the human. The erythrocytic cycle is responsible for the clinical manifestations of malaria. Adapted from the Centers for Disease Control and Prevention (CDC).

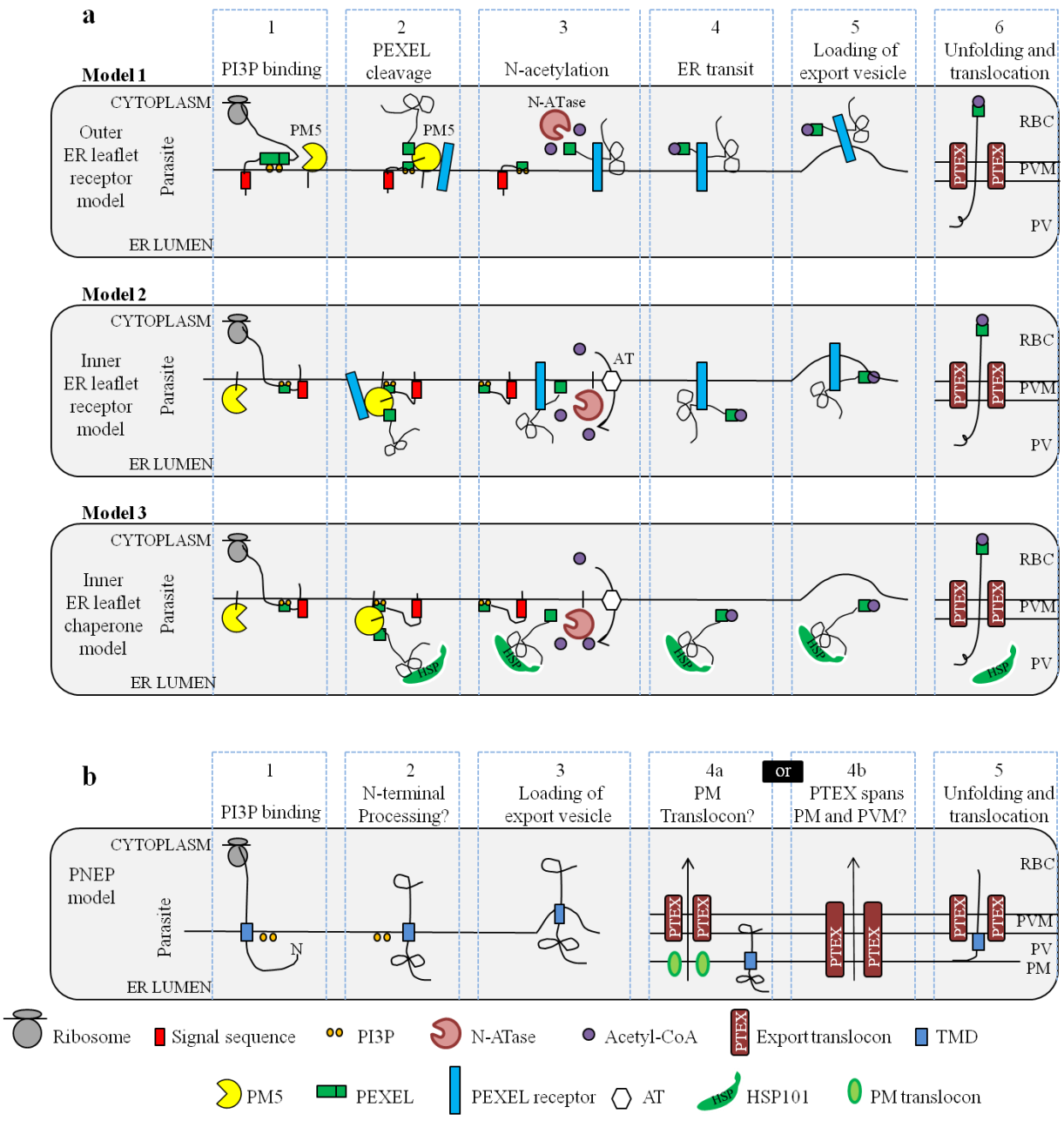

Figure 3. Models of Plasmodium protein export.

(a) Three models of PEXEL protein export. Model 1 illustrates PI3P binding, PEXEL cleavage by PM5, and N-acetylation of the mature protein on the outer leaflet of the ER, while these steps occur on the inner leaflet of the ER in Models 2 and 3. In Models 1 and 2, the mature protein is

recruited and escorted to the PTEX by a PEXEL receptor. In Model 3, the mature protein is recruited and escorted to the PTEX by Hsp101. **(b)** Model of PNEP export. PNEP proteins are targeted to the ER by a TMD. PI3P binding at the ER is unknown. PNEPs may or may not be cleaved by PM5 and N-acetylated. Abbreviations: AT, acetyl-CoA transporter; ER, endoplasmic reticulum; N-ATase, N-acetyltransferase; PEXEL, *Plasmodium* export element; PI3P, phosphatidylinositol-3-phosphate; PM, parasite membrane; PM5, Plasmepsin V; PNEP, PEXEL-negative exported protein; PTEX, *Plasmodium* translocon of exported proteins; PV, parasitophorous vacuole; PVM, parasitophorous vacuole membrane; REX, ring-exported protein 2; RBC, red blood cell; TMD, transmembrane domain. Adapted from (Boddey and Cowman, 2013).

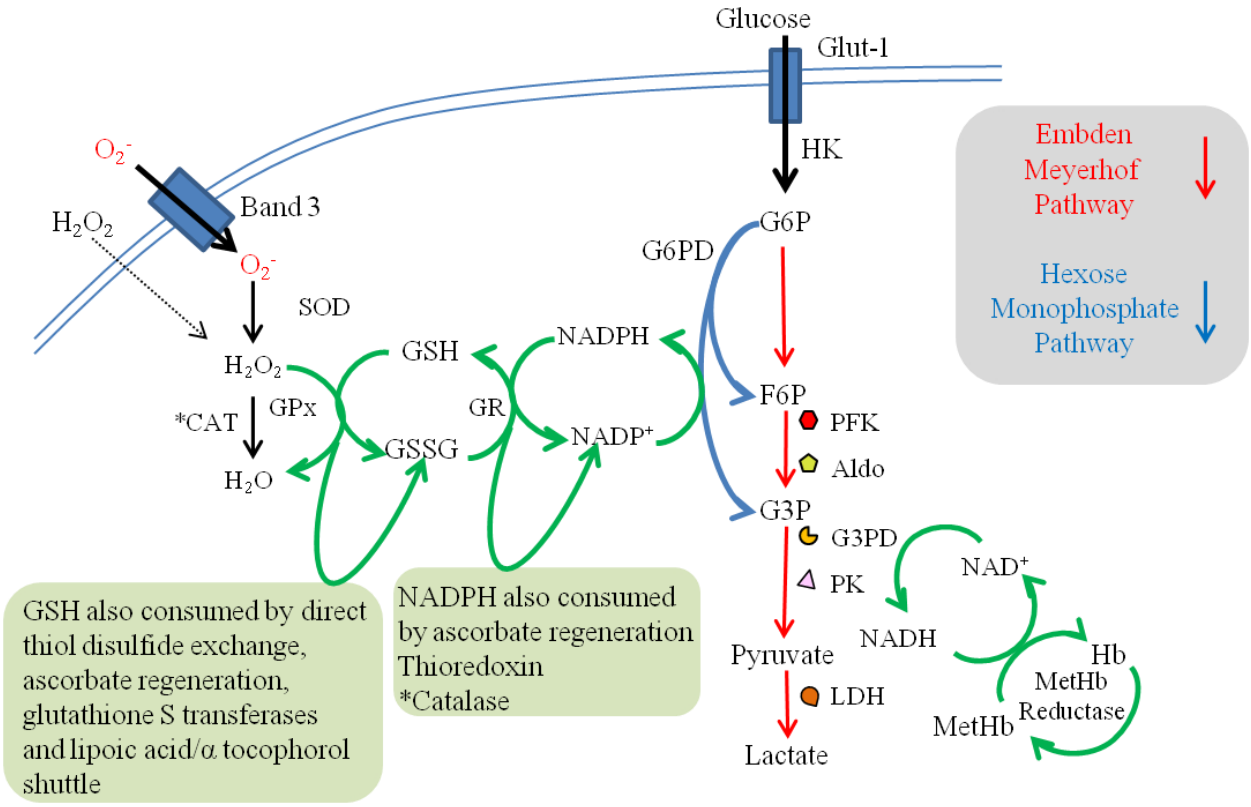

Figure 4. Scheme of glucose metabolism in erythrocytes.

Glucose is transported by Glut1 then converted to glucose-6-phosphate by hexokinase. G6P is used in glycolysis to produce ATP for energy and the pentose phosphate pathway to produce NADPH for antioxidant defense. Abbreviations: G6P, glucose-6-phosphate; G6PD, Glucose 6-phosphate dehydrogenase; GLUT1, glucose transporter; HK, hexokinase; GSH/GSSG, reduced/oxidized glutathione. Adapted from (Rogers *et al.*, 2009).

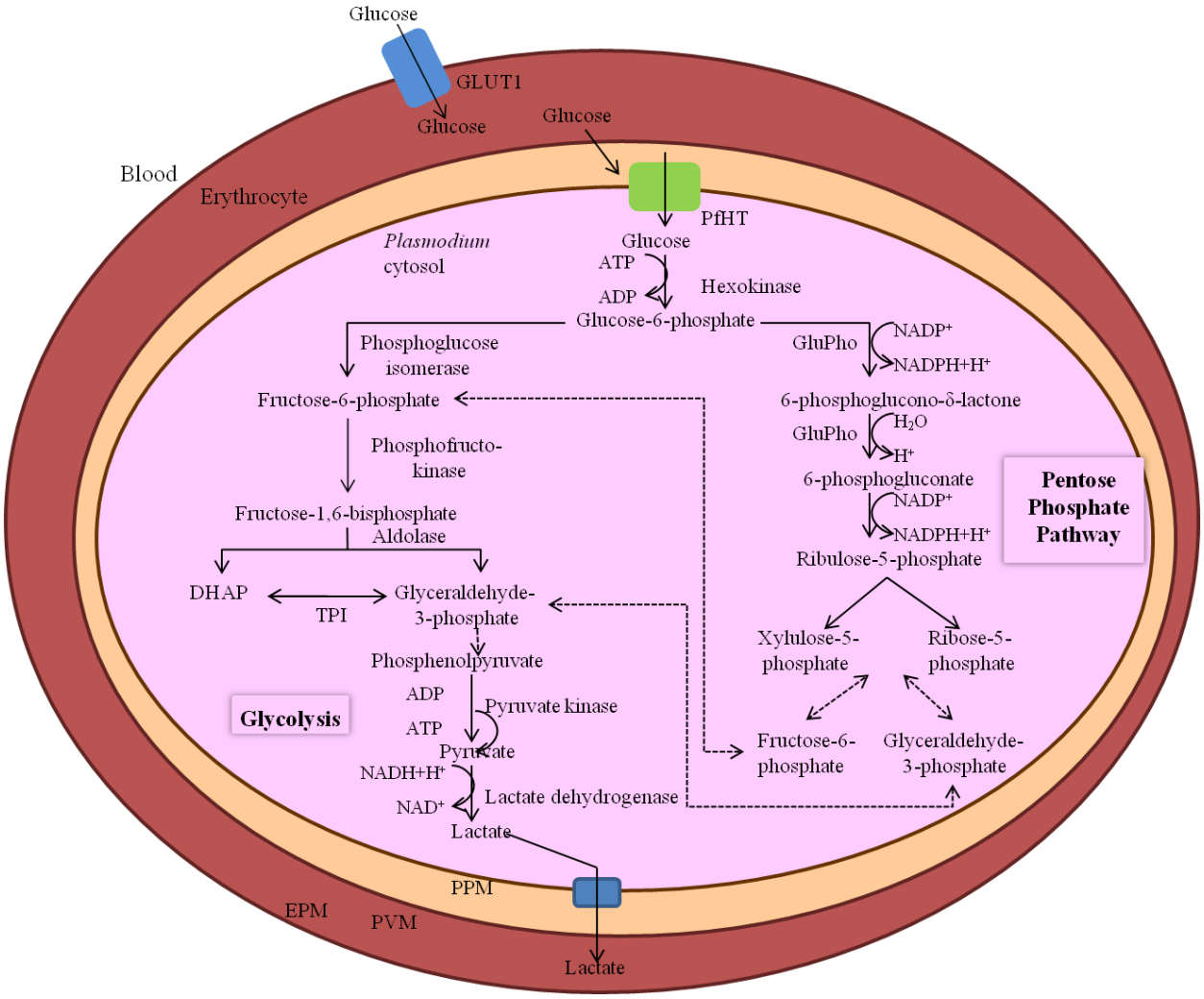

Figure 5. Scheme of glucose metabolism in *Plasmodium*.

Glucose is converted to glucose-6-phosphate which is used in glycolysis to produce ATP for energy and the pentose phosphate pathway to produce NADPH for antioxidant defense. Abbreviations: 6PGD, 6-phosphogluconate dehydrogenase; DHAP, dihydroxyacetone phosphate; EPM, erythrocyte plasma membrane; GluPho, glucose-6-phosphae dehydrogenase 6-phosphogluconolactonase; GLUT1, glucose transporter; PfHT, *P. falciparum* hexose transporter; PPM, parasite plasma membrane; PVM, parasitophorous vacuole membrane; TPI, triosephosphate isomerase. Adapted from (Preuss *et al.*, 2012).

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CHAPTER II

Malaria parasites subvert stomatin function to reestablish glucose uptake in infected erythrocytes

ABSTRACT

Intraerythrocytic malaria parasites are dependent on glucose as a nutrient source. *Plasmodium falciparum*-infected erythrocytes consume up to 100 times more glucose than uninfected erythrocytes, as both glycolysis and the pentose phosphate shunt are massively increased. How the parasitized red blood cell (RBC) obtains glucose from the blood stream is a mystery. The parasite does not use its own nutrient acquisition systems, relying instead on endogenous erythrocyte glucose transport. However, this does require rewiring by the parasite as erythrocyte glucose transport is dramatically downregulated during red blood cell maturation, via upregulation of stomatin. We report that a small, exported parasite protein that we call glucose uptake restoration protein (GURP) localizes with and sequesters stomatin to novel double membrane vesicles. Conditional knockdown of GURP decreases glucose uptake and impairs parasite viability. Following knockdown, parasite growth can be rescued by antioxidants, suggesting that glucose supply to the pentose phosphate shunt is rate limiting.

INTRODUCTION

Plasmodium falciparum, the protozoan parasite that causes the most lethal form of human malaria, extensively remodels the red blood cell (RBC) during infection. Infected RBCs display a number of altered physical characteristics including increased rigidity and adherence to endothelial cells (Leech *et al.*, 1984; Barnwell, 1989). These modifications are mediated by the export of hundreds of parasite proteins into the RBC (Marti *et al.*, 2005; Maier *et al.*, 2009).

Most parasite proteins are targeted for export via the Plasmodium Export Element (PEXEL) (Marti *et al.*, 2004; Hiller *et al.*, 2004). *P. falciparum* contains a PEXEL -based exportome of approximately 300-400 proteins (Sargeant *et al.*, 2006; van Ooij *et al.*, 2008). Approximately 75% of the exportome belongs to known protein families, leaving an estimated 100 proteins labeled as "exported protein, unknown function" (Sargeant *et al.*, 2006). Here, we demonstrate that an exported protein of unknown function, that we call GURP (PFB0923c), regulates glucose uptake during intraerythrocytic infection.

Plasmodium infected red blood cells (iRBCs) show a dramatic increase in glucose metabolism compared to uninfected red blood cells (uiRBCs); both glycolysis and the pentose phosphate pathway (PPP) are increased by nearly 100 fold (Roth *et al.*, 1988; Atamna *et al.*, 1994). Glycolysis is the main source of ATP in *Plasmodium* during the blood stage and as such, uptake and metabolism of glucose is critical for parasite survival (Preuss et al, 2012). The iRBC has elevated levels of PPP activity in the parasite compartment as well as the host erythrocyte compartment (Atamna *et al.*, 1994).

Erythroid precursors take up large amounts of glucose. As the erythrocyte matures, expression of stomatin is induced. This is believed to decrease glucose uptake by an order of magnitude (Zhang *et al.*, 2001), via an unknown mechanism. In order to satisfy its metabolic

demands, intraerythrocytic *P. falciparum* has to overcome stomatin-induced repression of erythrocyte glucose uptake. Interestingly, we find that stomatin localization is dramatically changed following erythrocyte infection. We show that GURP localizes with and sequesters stomatin in novel double membrane delimited vesicles. Conditional knockdown of GURP prevents stomatin relocalization and leads to decreased glucose uptake and severely impaired parasite growth. This growth defect can be rescued by antioxidants, suggesting that glucose supply to the PPP is rate limiting. Taken together, our data shows that GURP is essential for parasite development and glucose uptake during intraerythrocytic infection.

RESULTS

GURP is exported into the host RBC and localizes to a novel compartment

P. falciparum exports numerous proteins into the host RBC to remodel the cell for parasite survival during infection. GURP contains a protein export element (PEXEL) downstream of a signal sequence (Figure 1A), and has been computationally predicted to be exported from the parasite into the host RBC (Sargeant *et al.*, 2006). To assess export, antibodies raised against recombinant GURP protein (Figure 1B) were used in indirect immunofluorescence assays (IFA). GURP was detected in the RBC cytosol in discrete foci in asexual (Figure 1C) and sexual stage (Figure S1) parasites. Live fluorescence of parasites expressing a green fluorescent protein (GFP)-tagged GURP from the endogenous locus (described more fully in the context of Figure 3) gave similar results (Figure 1D). Staining with both anti-GURP and anti-GFP antibodies in parasites expressing the GFP-tagged GURP, show that GFP and GURP signals overlap (Figure 1E).

The punctate staining observed for GURP is reminiscent of that seen for two previously described infected erythrocyte structures, Maurer's clefts (MCs) (Blisnick *et al.*, 2000) and J-dots (Kulzer *et al.*, 2010; Kulzer *et al.*, 2012). To determine if GURP localized to either of these, co-localization studies were done with markers of MCs (skeleton-binding protein 1, SBP1) and J-dots (Hsp40, type II). Neither SBP1 nor Hsp40 overlapped with GURP, indicating that these structures are distinct from MCs and J-dots (Figure 2). Taken together, these data show that GURP is exported into the RBC cytosol where it localizes in a novel compartment.

GURP export is essential for parasite growth

To explore the function of GURP, we first tried to knock out the *GURP* gene by double crossover homologous recombination; however, several attempts were unsuccessful. We then

turned to a conditional knockdown system using the dihydrofolate reductase (DHFR) destabilization domain (DDD) (Muralidharan *et al.*, 2011; Muralidharan *et al.*, 2012). In the absence of the DHFR ligand trimethoprim (TMP), DDD fusion proteins are unstable and often targeted for degradation. In the presence of TMP, fusion proteins are stabilized. We integrated a C-terminal RFA (regulatable, fluorescent, affinity) tag at the genomic locus for GURP (Figure 3A). This tag encodes GFP fused to the DDD (Muralidharan *et al.*, 2011). The resulting integrants, upon protein expression, produce a fusion protein that can be used to follow protein in live cells (Figures 1D), to do protein pulldowns (Figure 5A, Table 1) and to regulate protein function (Figure 4A). Southern blot analysis of integrant clones (Figure 3B) showed replacement of the native GURP locus band with a higher band expected for integration of the RFA sequence at the open reading frame 3' end.

To assess knockdown of GURP, we grew parasites +/- TMP over time, magnet purified the mature iRBCs (Ribaut et al, 2007), and performed western blot analysis using anti-GURP antibodies. There was no significant decrease in GURP levels in the –TMP samples (Figure 3C). However, failure to export GURP was observed in cultures grown without TMP for 36 hours (Figure 3D). In contrast, iRBCs cultured in the presence of TMP exported the GURP fusion protein as expected (Figure 3D).

To determine the effect of impaired GURP export on parasite growth, we removed TMP and measured parasitemia over time by flow cytometry. A severe growth defect was apparent in parasites grown without TMP (Figure 4A). To ensure that the phenotype observed is specific to GURP functional knockdown and not due to non-specific effects of protein mislocalization on the cell, we complemented the conditional knockdown parasites with a plasmid for episomal expression of GURP. As before, we grew the parasites +/- TMP and measured parasitemia over

time by flow cytometry. The parasites carrying the GURP plasmid grew at wild-type levels in the absence of TMP, while those carrying the empty vector still grew at knockdown levels (Figure 4B). This phenomenon of a functional knockdown without a decrease in protein levels has been seen in previous work (Muralidharan *et al.*, 2012). Taken together, these data show that GURP is essential for parasite growth and development in RBCs.

GURP interacts and colocalizes with host protein stomatin

GURP has no identifiable orthologs or protein function motifs, though it has been found to be palmitoylated (Jones *et al.*, 2012). The gene is restricted to the *P. falciparum* species. To ascertain what GURP's function might be, we looked for interacting partners. To do this, we performed parallel immunoprecipitation (IP) studies on the parental parasite line using anti-GURP antibody and on RFA-tagged GURP parasites using an anti-GFP antibody. Samples were digested with trypsin and analyzed by mass spectrometry to identify interacting proteins. Table 1 shows proteins for which four or more peptides were detected in at least one of the IP samples. The host protein stomatin was identified in both IPs. To confirm the interaction between GURP and stomatin, reciprocal IPs with anti-GURP and anti- stomatin antibodies were performed for western blot analysis (Figure 5A). Stomatin was pulled down with anti-GURP and GURP was pulled down with anti-stomatin antibody, confirming the interaction. IPs with anti-catalase and anti-carbonic anhydrase antibodies failed to confirm these candidate interacting proteins (data not shown). Peptides corresponding to parasite glyceraldehyde 3-phosphate dehydrogenase were also seen in both IPs but this interaction was not pursued and its significance is unknown.

To determine if GURP and stomatin colocalize, immunofluorescence (IFA) and immuno-electron (IEM) microscopy were performed with anti-GURP and anti-stomatin antibodies (Figure 5B, C). The micrographs show that stomatin and GURP colocalize, thus further

corroborating their interaction. Interestingly, the distribution of stomatin is noticeably different in iRBCs compared with uiRBCs. Infection results in a punctate pattern by IFA and residence in double membrane-delimited structures by IEM. It is worth noting that this change in pattern is not seen for the host protein Glut1, which is the major erythrocyte glucose transporter (Figure S2). Previous studies have shown that stomatin is partially located in detergent-resistant membranes (DRMs) in uninfected RBCs (Murphy *et al.*, 2004). Fractionation of iRBCs and treatment with Triton X-100 showed that GURP resides in DRMs and stomatin has completely shifted to the DRM fraction in iRBCs (Figure 5D). Together, these results show that GURP binds to and colocalizes with stomatin in iRBCs and that stomatin relocalizes upon *P. falciparum* infection.

GURP sequesters stomatin in vesicles

Given that stomatin is relocalized to vesicles in iRBCs and GURP interacts with stomatin, we hypothesized that GURP is responsible for this redistribution. To test this, we performed IFA on GURP-RFA parasite cultures grown +/- TMP. In cultures grown without TMP, stomatin localization in iRBCs was indistinguishable from that of uiRBCs (Figure 6). These data show that GURP is responsible for stomatin localization to vesicles in iRBCs, and suggests a potential mechanism behind the increase in glucose uptake in *P. falciparum* iRBCs.

Functional GURP knockdown causes a decrease in glucose uptake

Stomatin suppresses glucose uptake in mature RBCs (Montel-Hagen *et al.*, 2008) and Clone 9 cells (Zhang *et al.*, 2001). However, previous studies have shown that *Plasmodium* iRBCs dramatically increase their glucose utilization compared to uiRBCs (Mehta *et al.*, 2006). Given that GURP sequesters stomatin, we hypothesized that GURP functional knockdown would affect glucose uptake. To test this, we measured radiolabelled 2-deoxyglucose (2-DOG) uptake

in GURP-RFA parasites. Cultures grown in the presence of TMP showed rapid accumulation of 2-DOG, whereas cultures grown without TMP for 48 hours, took up label much more slowly (Figure 7A). 2-DOG is taken in by the equilibrative transporter Glut1 and then gets trapped inside the cell by phosphorylation. Therefore, uptake differences can reflect transport or metabolism. When the non-metabolizable analog 3-O-methyl glucose was used instead, uptake was unchanged as both + and – TMP cultures reached equilibrium at similar rates (Figure 7B). Taken together, these data show that GURP function is important for glucose phosphorylation (metabolism), but not transport. The defect in 2-DOG uptake could be rescued by providing GURP on a plasmid to GURP-RFA parasites (Figure 7C).

Knockdown growth defect can be rescued by antioxidants

Given that glucose is important for NADPH production (Atamna *et al.*, 1994), in addition to ATP, we hypothesized that parasites could be under increased oxidative stress following block of GURP export due to lack of NADPH, resulting from decreased glucose uptake. To test this, we grew parasites +/-TMP and added either DMSO or a variety of antioxidants to the culture medium and measured parasitemia over time by flow cytometry. Knock down growth was restored to +TMP levels with the addition of antioxidants, confirming that the parasites are oxidatively stressed due to reduced glucose levels (Figure 8) and that this is the toxicity first seen with decreased glucose availability

DISCUSSION

Export of hundreds of proteins into the host red blood cell is essential to *Plasmodium* survival during the intraerythrocytic stage of infection. As a whole, 50% of the proteins encoded by the *Plasmodium* genome are "hypothetical proteins" (Balu, 2012). *P. falciparum* exports approximately 400 proteins into the RBC cytosol, 8% of its total proteome (Sargeant *et al.*, 2006). Twenty-five percent of these exported proteins are hypothetical, or "exported proteins, unknown function" (Sargeant, *et al.*, 2006). Investigations into the functions of these proteins are important as they may provide novel drug targets. Development of new drug treatments for malaria is important due to parasite resistance to current antimalarials (Mita and Tanabe, 2012).

In our work here, we have described an exported protein of unknown function, GURP. We have shown that GURP is indeed exported to the host RBC and localizes to double membrane vesicles. Co-immunofluorescence studies show that these are not components of the Maurer's clefts (Blisnick *et al.*, 2000) or J-dots (Kulzer *et al.*, 2010; Kulzer *et al.*, 2012) that have been implicated in parasite protein trafficking through the erythrocyte (Boddey, 2013)(Figure 2). We do not yet know what these vesicles are comprised of, but we believe that they are parasite derived, as these vesicles are not seen in uninfected RBCs. In our GURP knockdown we see that stomatin no longer localizes to vesicles (Figure 6), suggesting that GURP acts by sequestering stomatin in these structures. Both GURP and parasite-hijacked stomatin are detergent resistant (Figure 3). The physical basis for this property is unclear, but both proteins are known to be palmitoylated (Jones *et al.*, 2012; Snyers *et al.*, 1999), a post-translational modification shown to target proteins to membranes and vesicles for transport (Salaun *et al*, 2010).

We have shown that GURP is essential to parasite growth, as functional knockdown of GURP elicits a severe growth defect (Figure 4). Since GURP sequesters stomatin and stomatin is known to have an effect on glucose uptake, we measured uptake in the knockdown parasites and showed that it is profoundly reduced. We propose, then, that GURP sequestration of stomatin allows the parasite to change its host cell back to a state of high glucose acquisition, similar to that seen in the low-stomatin erythroid precursors. Stomatin has been postulated to alter Glut1-mediated glucose transport in mature erythrocytes (Montel-Hagen *et al.*, 2008), though these findings have been disputed (Carruthers and Naftalin, 2009). Glucose trapping by phosphorylation upon entry into the erythrocyte has been posited as an alternative interpretation. In P. falciparum-infected erythrocytes, the reduction is a result of impaired phosphorylation rather than transport. This suggests impaired function of the host glycolytic enzyme hexokinase which catalyzes the phosphorylation of glucose to glucose-6-phosphate, which is a substrate for glycolysis and the pentose phosphate shunt. Interestingly, hexokinase activity is downregulated during erythrocyte maturation (Shinohara *et al.*, 1985) but upregulated in *P. falciparum* iRBCs (Roth, 1987; Roth *et al.*, 1988). Our data can therefore link together all these disparate observations. To determine if GURP knockdown affects hexokinase activity, we will need to perform detailed measurements of hexokinase activity in fractionated GURP-RFA parasites that have been grown with and without TMP.

Decreased glucose acquisition in the functional GURP knockdown parasites has a drastic effect on parasite growth. While glycolysis generates ATP for the parasite to use as energy, the pentose phosphate shunt produces antioxidants, including NADPH and reduced glutathione, which are also important. As the parasite develops, large amounts of toxic redox-active waste are generated, mostly from digestion of host hemoglobin (Becker *et al.*, 2004). The parasite uses

hemoglobin as its source of amino acids, but its degradation leads to formation of the toxic free heme and reactive oxygen species. Free heme is mostly detoxified by packing in to hemozoin crystals (Egan *et al.*, 2002; Slater and Cerami, 1992), however, there is no evidence that all heme is sequestered and it could cause oxidative stress to the parasite (Tilley *et al.*, 2001). Chloroquine functions by preventing heme detoxification and its activity can be enhanced y depletion of GSH (Tilley *et al.*, 2001). This illustrates the importance of antioxidants to the parasite during intraerythrocytic development. There is also substantial oxidative stress to the host erythrocyte. If RBC NADPH generation is limited, as in G6PD deficient RBCs, parasite survival is impaired (Luzzatto et al, 1969). That we can rescue the GURP knockdown growth defect by supplying exogenous antioxidants (Figure 8), indicates that the pentose phosphate shunt is limiting in iRBCs and that the knockdown growth defect is due to oxidative stress stemming from the deficiency in glucose uptake. Whether this is due to a limitation in the host cell or the parasite antioxidant capacity remains to be established.

Further study of GURP-stomatin interaction could provide information necessary to design specific GURP inhibitors. Drugs that prevent GURP binding to and sequestering stomatin may act as potent antimalarials.

MATERIALS AND METHODS

DNA Sequences and Cloning

The GURP open reading frame was amplified by PCR from *P. falciparum* genomic DNA using the primers listed in Table S1. The PCR product was then cloned into the pCR4-TOPO vector (Life Technologies, Grand Island, New York) and sequenced. An internal AvrII site was disrupted by introducing a synonymous mutation (GURPmut-TOPO) using the primers listed in Table S1 and the QuickChange XL Site-Directed Mutagenesis Kit (Stratagene, Santa Clara, California). A 561 bp sequence from the 3' end of the *gurp* ORF was PCR amplified from GURPmut-TOPO using primers listed in Table S1. To construct the RFA tag vector, this 3' homologous sequence was inserted into the GDB vector (Muralidharan *et al.*, 2011) via XhoI/AvrII restriction sites using the In-Fusion cloning system (Clontech, Mountain View, California). For plasmid complementation, GURP cDNA was amplified by RT-PCR (SuperScript III One-Step RT-PCR, Life Technologies) from *P. falciparum* RNA using the primers listed in Table S1, spanning the entire ORF, to bypass an 189 base pair intron near the 5'end of the gene. GURP cDNA was cloned into the pCR4-TOPO vector (Life Technologies) and sequenced. The internal AvrII site was mutated as above. XhoI and AvrII restriction sites were used to insert GURP cDNA into ptagRFP-T (Muralidharan *et al.*, 2012) by overnight ligation using T4 DNA Ligase (Roche, Basel, Switzerland) at 16°C. The PFE0055c (Hsp40, type II) open reading frame was amplified by RT-PCR from *P. falciparum* total RNA using the primers listed in Table S1. The cDNA product was then cloned into the tyEOE vector (Muralidharan *et al.*, 2012) via XhoI and AvrII restriction sites using the In-Fusion cloning system (Clontech), resulting in a C-terminal GFP tag fusion under control of the Hsp86 promoter.

Cell Culture and Transfections

Parasites were cultured in RPMI medium supplemented with Albumax and transfected as described previously (Drew *et al.*, 2008; Russo *et al.*, 2009). Parasites with a hDHFR resistance cassette inserted at the Plasmepsin I locus (Klemba *et al.*, 2004; Muralidharan *et al.*, 2011) were transfected with pGURP-GDB. After 48h they were selected with 2.5 μg/mL blasticidin (Millipore, Darmstadt, Germany) and 10 μM TMP (Sigma, St. Louis, Missouri) (Muralidharan *et al.*, 2011; Muralidharan et al, 2012). TMP was re-added with every change of culture medium. Integration was detected after two rounds of cycling on blasticidin for 14 days and off blasticidin for 21 days (Wu *et al.*, 1996). Clones were isolated via limiting dilution (Francois *et al.*, 1994). For plasmid complementation, GURP-GDB parasites were transfected with pGURP-tagRFP-T or ptagRFP-T which contains a yeast dihydroorotate dehydrogenase selection marker allowing selection episome maintenance using 2 μM DSM-1 (Ganesan *et al.*, 2011). Greater than 90% of parasites displayed red fluorescence, indicating they were carrying the plasmid. Cultures underwent positive selection after 48h with 2.5 μg/mL BSD, 10 μM TMP, and 2 μM DSM-1. For knockdown assays, GURP-GDB parasites were washed three times in RPMI 1640 media with 2.5 μg/mL blasticidin to remove trimethoprim (TMP). Washed cultures were then equally divided into two separate samples, 2.5 μg/mL blasticidin/10 μM TMP (+TMP) was added to one sample while blasticidin only medium was added to the other (-TMP). For antioxidant rescue, either DMSO alone, 0.4 μM vitamin C (vitC), 5 mM N-acetyl-L-cysteine (NAC), or 10 μM reduced glutathione (GSH) was added to +/- TMP cultures. All cultures were plated in triplicate in 1mL volumes for flow cytometry. For IFA of non-transfected parasites and for Hsp40 transfections, we used clone D10 parasites.

GURP Protein Expression and Antibody Production

GURP cDNA encoding the predicted mature protein after PEXEL processing (GURPmat), was amplified by PCR from GURPcDNA-pCR4-Topo using the primers listed in Table S1. The cDNA was cloned into the pCR4-TOPO vector (Life Technologies), sequenced, and then inserted into the pET28a vector (Novagen, Darmstadt, Germany) using BamHI and EcoRI restriction sites by overnight ligation. The plasmid was transformed into C41(DE3) cells (Lucigen, Middleton, Wisconsin) for protein expression. Two liters of YT medium was inoculated with overnight culture to OD_{600}=0.1. Cultures were shaken at 37°C to OD_{600}=0.6 then induced with isopropyl β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 1 mM for four hours. Cells were harvested, protein was extracted from inclusion bodies with 8M Guanidine-HCl (Palmer and Wingfield, 2004) and purified over His-Bind, nickel-charged resin (Novagen). Antiserum was raised against purified protein in a rabbit (Cocalico Biologicals, Inc., Reamstown, Pennsylvania).

Immunoprecipitation

MACS-purified iRBCs and uiRBCs were resuspended in 500 μL radioimmunoprecipitation assay (RIPA) buffer. Cell suspensions were freeze/thawed three times on dry ice/37°C water bath then centrifuged for 3 minutes at 800 *g*, 4°C in an eppendorf 5415 table top centrifuge to make lysates (Armstong and Goldberg, 2007; Dvorin et al, 2010; Muralidharan *et al.*, 2011). 50 μL of Protein G-linked Dynabeads (Life Technologies) were incubated for 30 minutes with 0.2 μg mouse monoclonal anti-GFP, 3E6 (Life Technologies) or rabbit anti-GURP. The bead-antibody complex was then incubated with the uiRBC or iRBC lysates for 4 hours at 4°C. The beads were washed three times with PBS and resuspended in 50 μL of SDS-PAGE loading buffer. The samples were fractionated by 10% SDS-PAGE and

vertical strips from each lane were cut out and analyzed by 1D-nLC-ESI-MS-MS (Dundee Proteomics Facility, Scotland, United Kingdom) for identification of proteins. Reverse immunoprecipitation was done as above but with mouse monoclonal anti-GFP, 3E6 or rabbit anti-stomatin (Santa Cruz Biotechnology, Inc., Dallas, Texas). Samples were resuspended in 4x non-reducing SDS-PAGE sample buffer and subjected to 10% SDS-PAGE for Western blot analysis. Control IPs using parasites expressing GFP only were used to eliminate non-specific binding partners (Muralidharan *et al.*, 2012).

Western Blot

Samples separated by SDS-PAGE were transferred to nitrocellulose membrane (Fisher Scientific, Pittsburgh, Pennsylvania) and incubated for 1 hour in blocking buffer (LI-COR Biosciences, Lincoln, Nebraska). The primary antibodies used were goat anti-stomatin (1:1000) (Santa Cruz Biotechnology, Inc.), rabbit anti-GFP (1:1000) (Abcam, Cambridge, Massachusetts), rabbit anti-GURP (1:500), mouse anti-HRP2 (2G12) (1:2000) (Diane Taylor) and mouse anti-glycophorin A (1:1000) (BD Biosciences, San Jose, California). The primary antibodies were detected using IRDye 680 CW (1: 10000) conjugated donkey anti-rabbit (LI-COR Biosciences) and IRDye 800CW (1:10000) conjugated donkey anti-mouse or anti-goat (LI-COR Biosciences). The western blot images were processed and analyzed using the Odyssey infrared imaging system software (LI-COR Biosciences). For testing rabbit anti-GURP specificity, samples were handled as above except the membrane was blocked in 5% BSA in PBS and the secondary antibody was a donkey ECL™ anti-rabbit HRP conjugate (GE Healthcare, Buckinghamshire, United Kingdom). Bands were visualized with ECL™ Western Detection Kit (GE Healthcare) and exposure to film.

Indirect Immunofluorescence Assay (IFA) and Live Cell Imaging

IFA was done as described previously (McMorran *et al.*, 2012). Briefly, thin blood smears prepared from parasite cultures were air-dried, fixed in methanol for 30 seconds, then fixed in 1% paraformaldehyde in PBS for 20 minutes. Smears were washed in PBS then blocked and permeabilized with 1% BSA/0.05% Triton X-100 in PBS. Goat anti-stomatin (1:100) (Santa Cruz Biotechnologies, Inc.), rabbit anti-GURP (1:250), rabbit anti-GLUT1 (1:500) (WU674) (Mike Mueckler) or rat anti-SBP1 (1:200) (Catherine Braun-Breton) primary antibodies were added for 1 hour. Smears were washed 3 times in PBS then incubated with Alexa Fluor™555 donkey anti-rabbit (1:500) and Alexa Fluor™ 488 chicken anti-goat or rat (1:500) secondary antibody conjugates. Smears were washed again then mounted in ProLong Gold with DAPI (Life Technologies). Live parasites were stained with 2 μM Hoeschst 33342 (Life Technologies) as described previously (Klemba et al, 2004). Cells were observed on an Axioscope Microscope (Carl Zeiss Microimaging, Oberkochen, Germany). Deconvolutions were performed on Z-stack images using AxioVision software.

Southern Blots

Southern blots were performed with genomic DNA isolated using the QIAmp® Blood DNA Mini Kit. One μg of DNA was digested overnight with EarI and Avr II (New England Biolabs, Ipswich, Massachusetts). Digested DNA was precipitated and concentration was determined by A280 using a Nanodrop. Integrants were screened using a probe against the 3' homologous region used for integration. All Southern blots were performed as described previously (Klemba *et al.*, 2004).

Flow Cytometry

Aliquots of parasite cultures (7 μL) were stained with 1.5 μg/mL Acridine Orange (Life Technologies) in PBS (Muralidharan *et al.*, 2012). The fluorescence profiles of infected erythrocytes were analyzed by flow cytometry on a BD FACS Canto (BD Biosciences). The parasitemia data were fit to a standard exponential growth curve in the software package GraphPad Prism v 5.

Uptake Assays

MACS®-purified iRBCs (Ribaut *et al.*, 2007) (> 90% parasitized) were resuspended in PBS and a cell count (1-2.5 x 10^7 cells/mL) was taken on a MACSQuant Analyzer (Miltenyi Biotec, San Diego, California). Cells were resuspended in 1.5 mL of fresh PBS and incubated at room temperature for 20 minutes to deplete glucose. Cells were washed and resuspended in PBS and 100 μL aliquots of cells were placed in 1.5 mL eppendorf tubes. For glucose uptake, a 200 μL aliquot of solution containing 1.66 μCi/mL of 2-deoxy-D[1,2-^{3}H]glucose (DOG, Perkin-Elmer,Waltham, Massachusetts, 7.5 Ci/mmol) or 0.5 μCi/mL of methyl-D-glucose, 3-O-[methyl-^{14}C] (3-OMG, Perkin-Elmer,56.4 mCi/mmol) alone or supplemented with 5 mM unlabelled glucose, was added to the cells at 25 °C. For dehydroascorbic acid uptake, a solution containing 0.6 μCi/mL of L-[1-^{14}C]-ascorbic acid (Perkin-Elmer, 7.6 mCi/mmol) with 2 μL ascorbate oxidase (Millipore, 1U/μL) was incubated for 15 minutes to allow conversion of ascorbic acid to dehydroascorbic acid. A 200 μL aliquot of this solution either alone or supplemented with 20 mM unlabelled DHA, was added to the cells. Uptake was terminated by addition of ice-cold PBS + 50 μM CytB at various timepoints. Cells were centrifuged at 2000 *g* for 3 minutes, 4°C in an eppendorf 5415 table top centrifuge and 10 μL of supernatant was taken and mixed with scintillation fluid (Cloherty *et al.*, 1996; Kirk *et al.*, 1996; Vera *et al.*, 1996). The remaining

supernatant was aspirated and cells were washed twice in ice-cold PBS. Cells were lysed in 150 μL dH_20, bleached by addition of 150 μL of 6% perchloric acid, and centrifuged at 2000 *g* for 3 minutes to pellet debris. The supernatant was removed to scintillation vials and counted in a Beckman LS230 scintillation counter. Zero-time points were prepared by adding ice-cold PBS to cells before addition of radiolabelled solution. All time points were done in triplicate and each experiment was performed three times. Samples measured at time zero were averaged and subtracted from all other timepoint-averaged counts. Distribution ratio was calculated (Bonventre and Imhoff, 1970) and fit to a one-phase exponential association equation in the software package GraphPad Prism v5.

Ghost Membrane Preparation and Fractionation

For subcellular fractionation, MACS®-purified iRBCs (Ribaut et al, 2007) were incubated for 15 minutes at 4°C in 40 volumes of hypotonic solution (RPMI 1640 diluted 1/5) with complete protease inhibitor cocktail (Roche) (Blisnick *et al.*, 2000). The lysates were centrifuged for 5 minutes at 800 *g*, 4°C in an Eppendorf 5415R table top centrifuge to pellet parasites. The supernatants were centrifuged for 1 hour at 109,000 *g*, 4°C in a Beckman Optima XL ultracentrifuge (Brea, California) to separate soluble and insoluble fractions. The insoluble (pellet) fraction was resuspended by trituration and treated with 1% Triton X-100, 150 mM $NaPO_4$, pH 7.4 for 30 minutes at 4°C then centrifuged at 109,000 *g* to separate detergent soluble and insoluble fractions (Sam-Yellowe *et al.*, 2001). All samples were resuspended in 4x reducing SDS-PAGE sample buffer, boiled, and subjected to 10% SDS-PAGE and analyzed by Western blot.

ACKNOWLEDGMENTS

We thank Akhil Vaidya (Drexel) for providing DSM-1, Natalie Spillman (Washington University in St. Louis) for discussion and help with uptake assays, Paul Hruz (Washington University in St. Louis) for discussion and help with uptake assays, Allan Doctor (Washington University in St. Louis) for helpful discussion and suggestions, Wandy Beatty (Washington University in St. Louis) for performing the immunoelectron microscopy, Josh Beck (Washington University in St. Louis) for help with microscopy, Mike Mueckler (Washington University in St. Louis) for Glut1 antibody, Catherine Braun-Breton (Istituto Superiore di Sanita (ISS), Rome, Italy) for Maurer's Cleft antibodies, Diane Taylor (University of Hawaii) for HRP2 antibody. We thank D. Lamont and K. Beattie (Dundee Proteomics Facility) for performing the mass spectrometry. We also thank Vasant Muralidharan (University of Georgia) and Anna Oksman (Washington University in St. Louis) for help with procedures and helpful discussion.

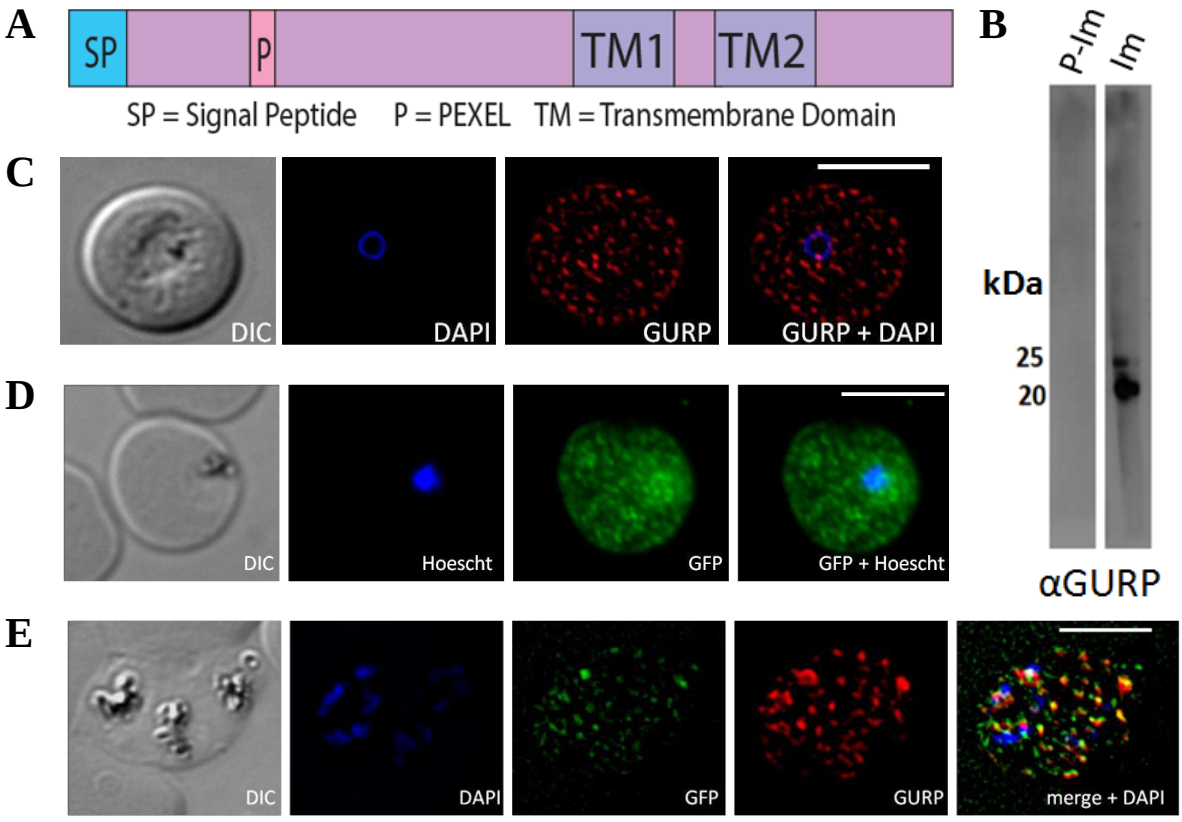


Figure 1. GURP is exported to the host RBC. (A) Schematic of GURP protein. **(B)** Western blot of rabbit anti-GURP antibody specificity. A band at the predicted mature 20 kDa mass is seen. A small amount of signal at the predicted mass of 25 kDa is also detected. **(C)** Deconvoluted Z-stack image of *P. falciparum* infected RBCs probed with anti-GURP antibody and DAPI nuclear stain. **(D)** Live image of a parasite expressing GURP-GFP and stained with Hoescht nuclear dye. **(E)** Deconvoluted Z-stack image of GURP-GFP expressing parasite probed with anti-GURP and anti-GFP antibodies. Scale bar = 5 μm. Abbreviations: P-Im, Pre-Immune serum; Im, Immune serum.

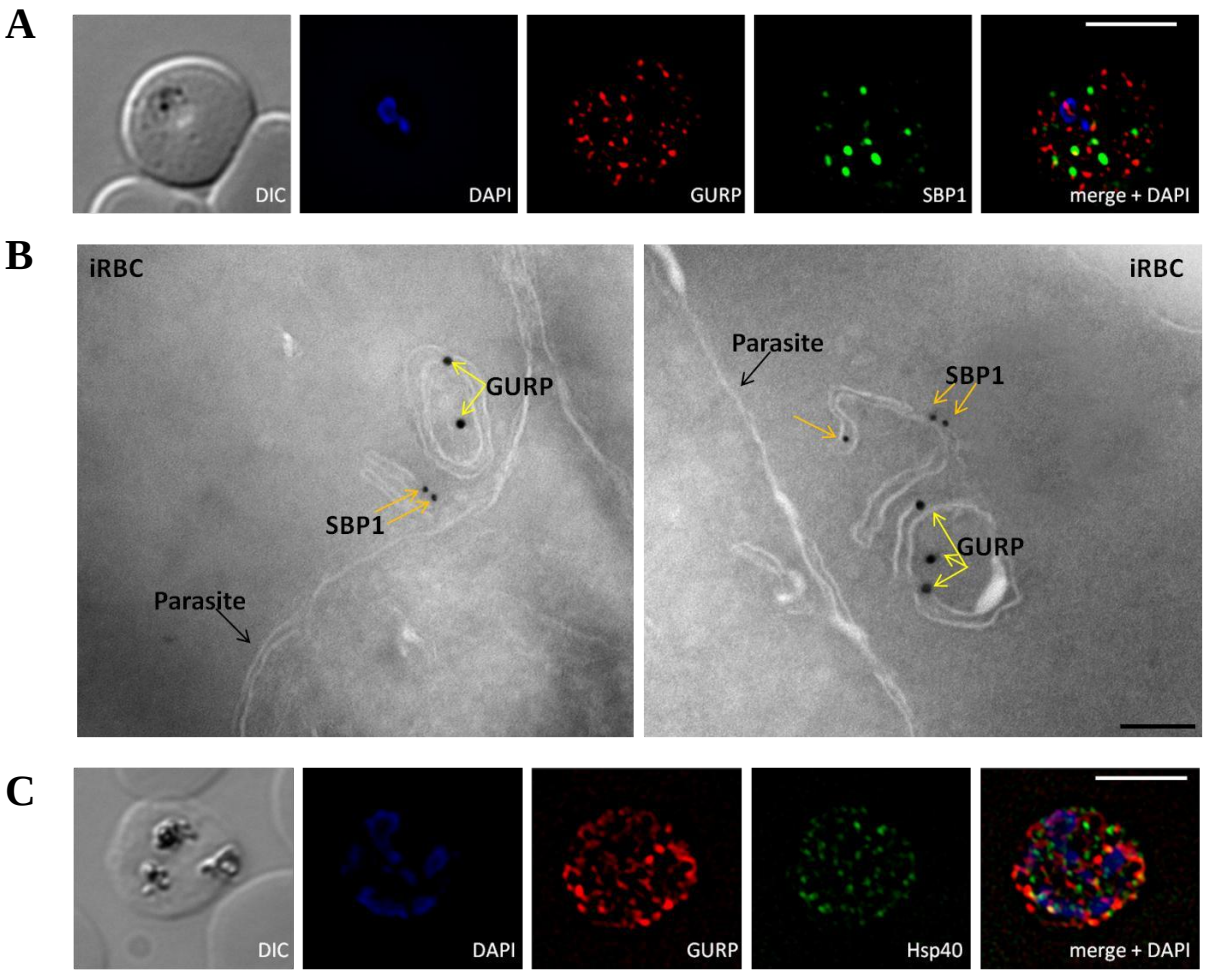


Figure 2. GURP localizes to compartments distinct from Maurer's clefts or J-dots. (A) Deconvoluted Z-stack images or **(B)** immuno-electron micrographs of *P. falciparum* iRBCs probed with anti-GURP and anti-SBP1 antibodies. **(C)** Deconvoluted Z-stack images of parasites expressing GFP-tagged Hsp40, probed with anti-GFP and anti-GURP antibodies. Abbreviations: SBP1, skeletal binding protein 1. A, C: Scale bar = 5 μm. B: Scale bar = 100 nm. 18 nm, yellow arrows = GURP; 12 nm, orange arrows = SBP1

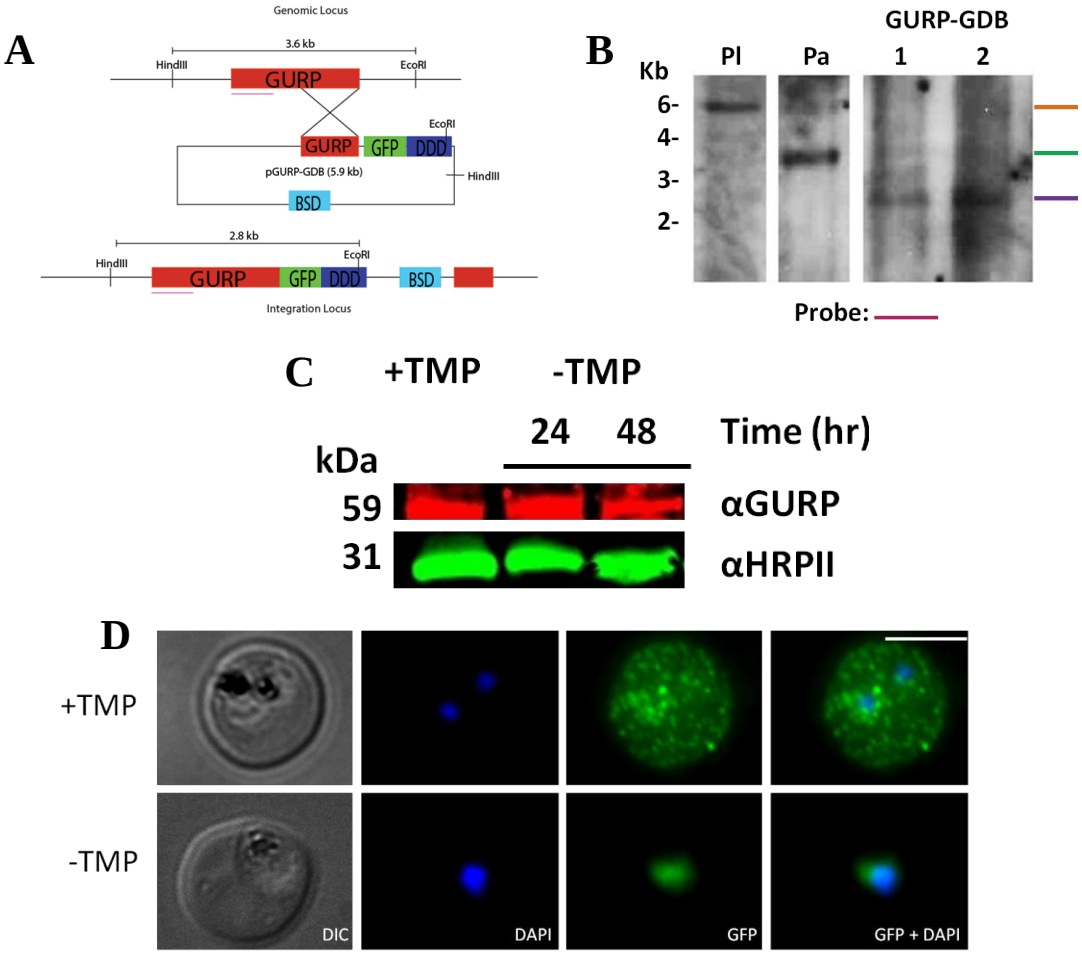


Figure 3. GURP export is knocked down in the absence of TMP.

(A) Scheme showing the strategy utilized to fuse the RFA-tag to the 3' end of the GURP gene via single crossover homologous recombination. **(B)** Southern blot of genomic and plasmid DNA digested with an EcoRI/HindIII double digest and probed with the 5' region of the GURP gene. The band expected for integration of the RFA-tag was seen in all clones (—). Bands for the plasmid (—) and parental strain (—) were not seen in any of the clones. **(C)** Western blot of

cultures grown +/- TMP, lysed, and probed with anti-GURP and anti-HRP2 antibodies. S=supernatant, P=pellet. **(D)** IFA of parasites grown +/- TMP for 36 hrs and probed with anti-GFP antibody. Scale bar = 5 μm. Abbreviations: Pl, Plasmid; Pa, Parent.

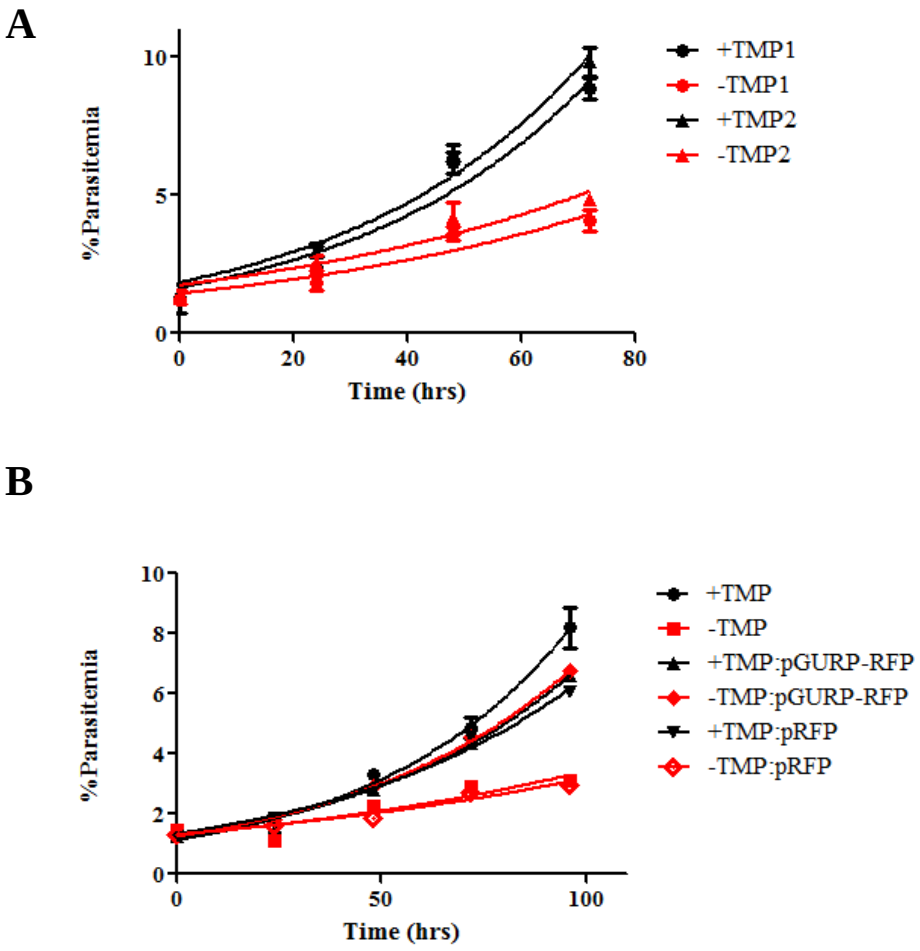


Figure 4. Functional knock down of GURP causes a growth defect that is rescued by plasmid complementation. (A) Two independent GURP-RFA parasite clones (clone 1, circles; clone 2, triangles) were grown with (black) or without (red) 10 uM TMP and growth was monitored over 4 days via flow cytometry. Data are fit to an exponential growth equation and are represented as mean ± S. E. M. (n=3). **(B)** Asynchronous GURP-GBD parasites complemented

with an episomal plasmid with the GURP gene (pGURP-RFP) or an empty vector (pRFP), were grown with (black) or without (red) 10uM TMP. Growth was monitored via flow cytometry. Data are fit to an exponential growth equation and are represented as mean ± S. E. M. (n=3). Shown are representative experiments from 3 biological replicates, each sample done in triplicate

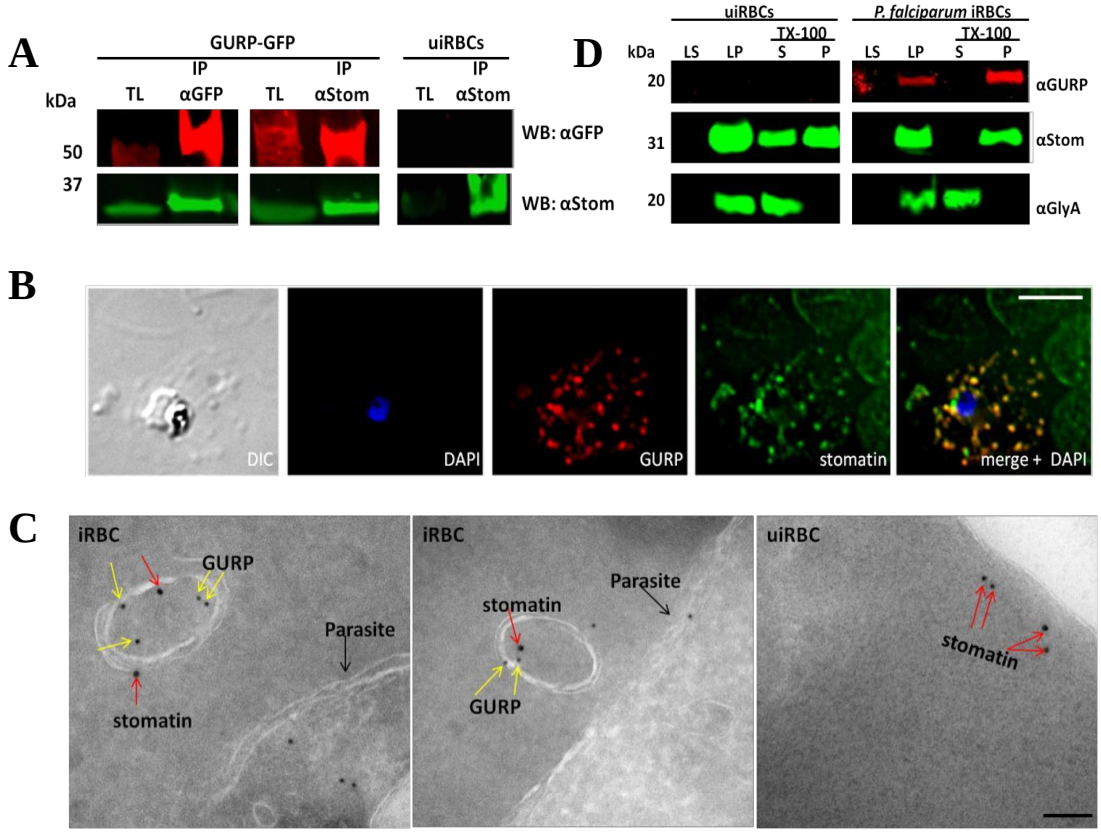


Figure 5. GURP interacts and colocalizes with stomatin in DRMs. (A) IP with anti-GFP and anti-stomatin (Stom) antibodies using parasites expressing GFP-tagged GURP. GURP and stomatin were detected by immunoblotting after SDS-PAGE. **(B)** Deconvoluted Z-stack images of *P. falciparum* iRBCs probed with anti-GURP and anti-stomatin antibodies. **(C)** Immuno-electron microscopy of *P. falciparum* iRBCs or uiRBCs probed with anti-GURP (12nm, yellow arrows) and anti-stomatin (18nm, red arrows) antibodies. **(D)** Fractionation and Triton X-100 extraction of *P. falciparum* iRBCs. GURP, stomatin (Stom), and

glycophorin A (GlyA, a control integral membrane protein) were detected by immunoblotting after SDS-PAGE. Abbreviations: TL, total lysate; LS, lysate supernatant; LP, lysate pellet; S, TX-100 soluble; P, TX-100 insoluble; iRBC, infected red blood cell; uiRBC, uninfected red blood cell. B: Scale bar = 5 μm. C: Scale bar = 100 nm.

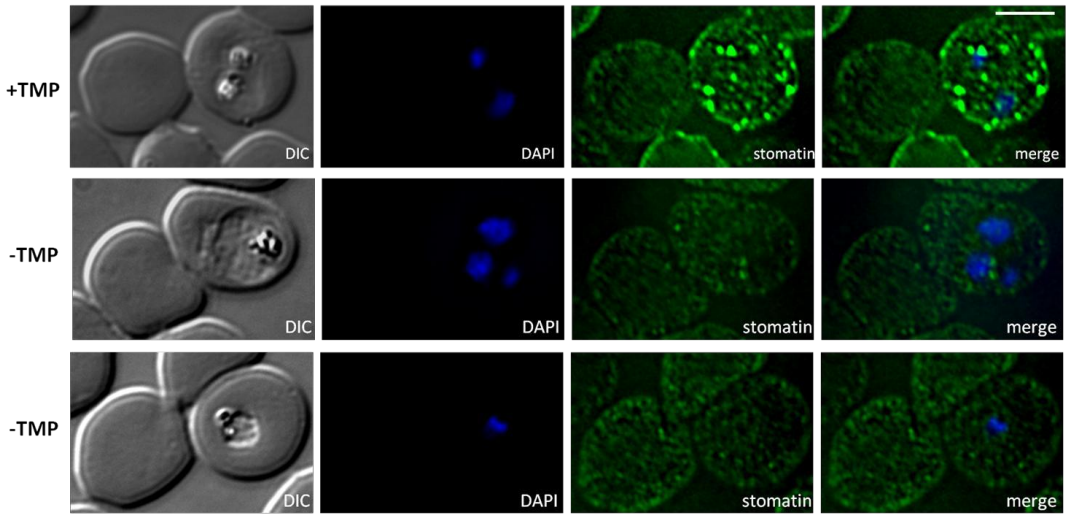


Figure 6. GURP sequesters stomatin in vesicles. Deconvoluted Z-stack images from IFA of parasites grown +/- 10 μM TMP for 48 hrs and probed with anti-stomatin antibody. Scale bar = 5 μm.

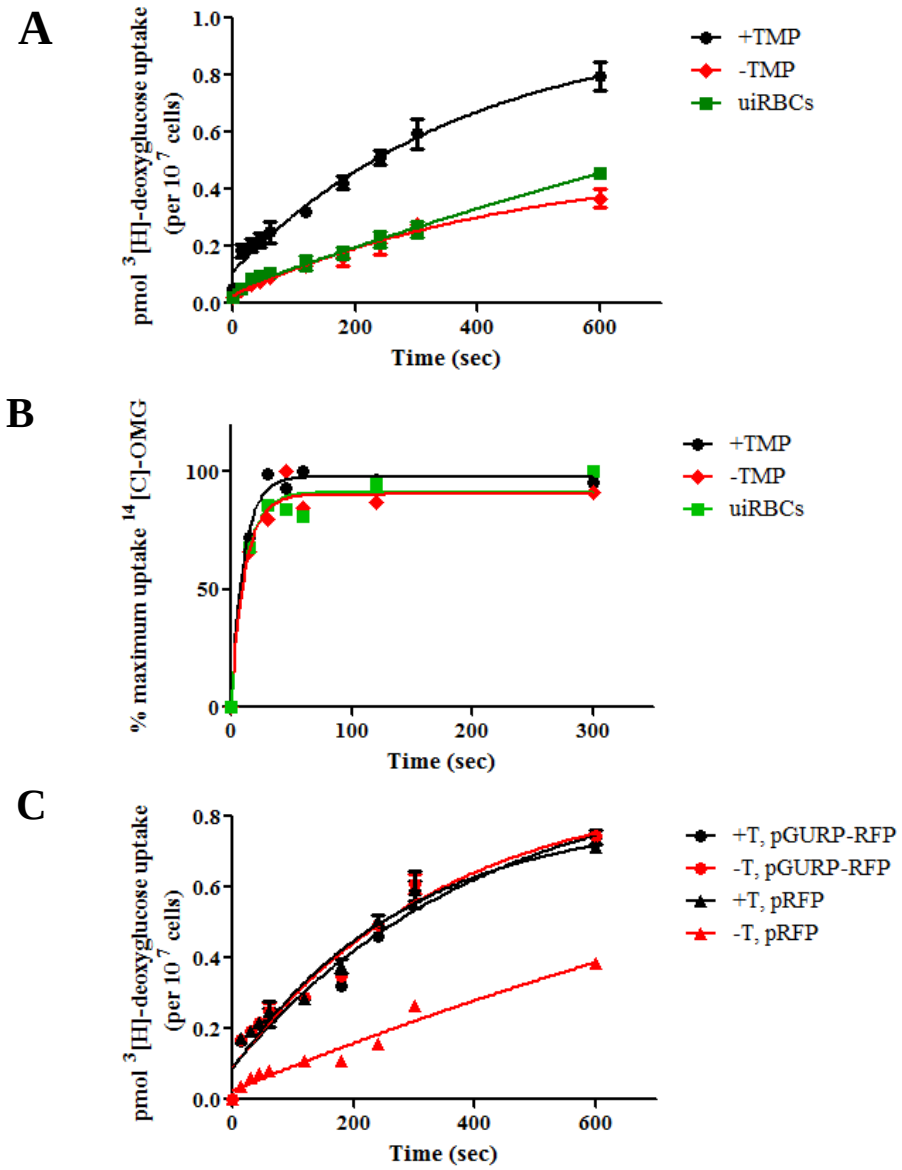


Figure 7. Functional knockdown of GURP causes a decrease in glucose uptake.

(A) Asynchronous GURP-GDB parasite clone was grown with (black) or without (red) 10 uM TMP for 48 hours and incubated with 2-deoxy-D[1,2-^{3}H]glucose. Samples were taken over time and uptake was plotted as pmol per 10^{7} cells. **(B)** Same as **(A)** but incubated with 3-O-[methyl-^{14}C]-glucose. Samples were taken over time and radioactivity uptake was quantified. Results are plotted as fraction of maximum uptake. Amount of uptake at equilibrium was approximately 1.6 pmol per 10^{7} cells, reaching a distribution ratio (OMG_{in}/OMG_{out}) of 1.0. **(C)** Same as **(A)** but with plasmid complemented parasites. Results are shown as the average of three biological replicates. Abbreviations: T, TMP; G, 10 mM unlabelled glucose added as competition.

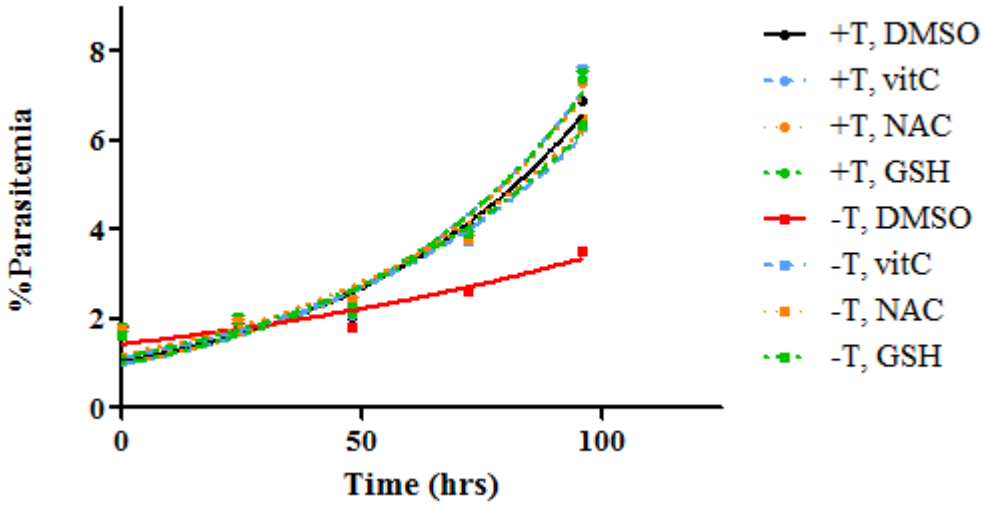
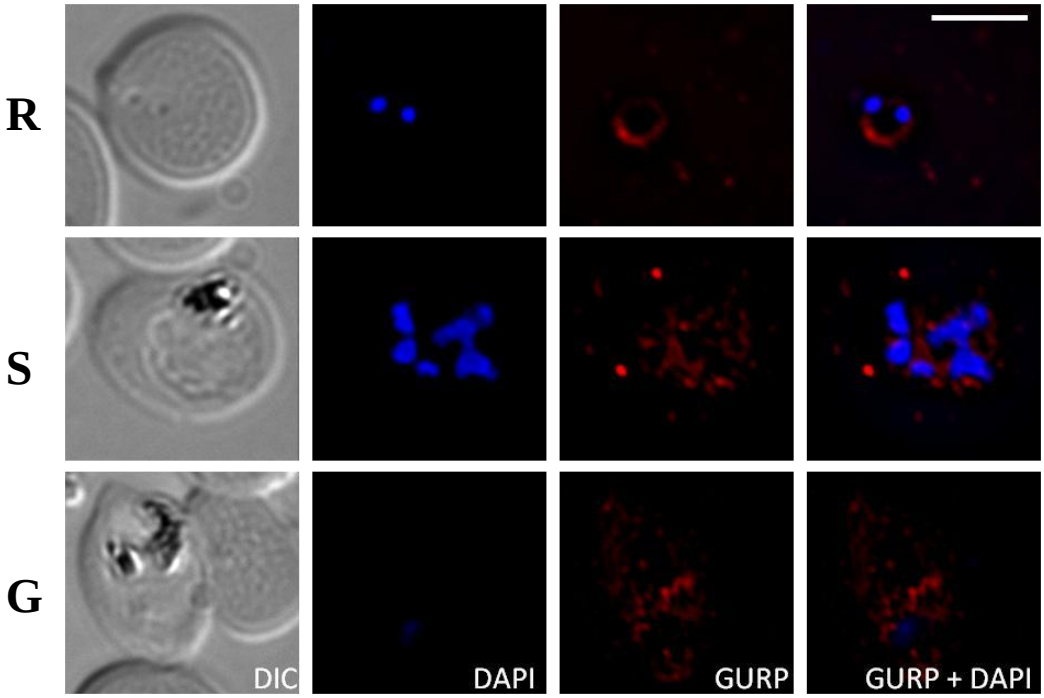


Figure 8. Antioxidants rescue knockdown growth phenotype.

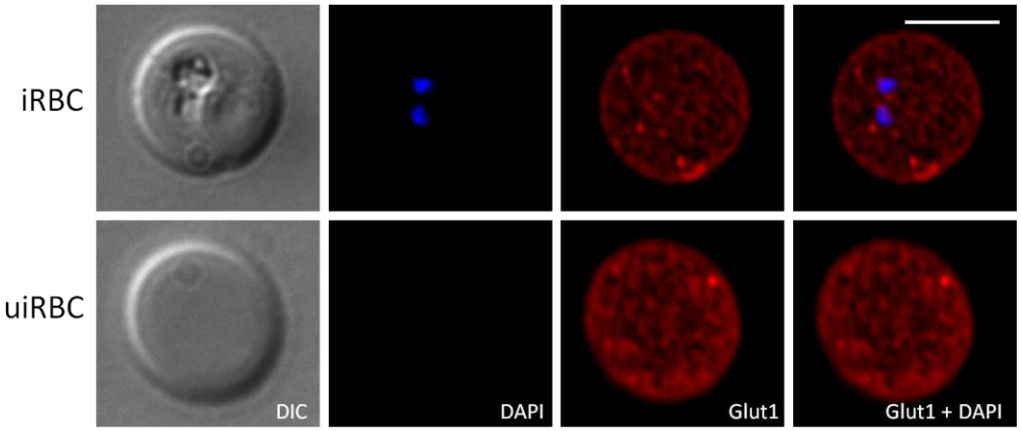
Asynchronous GURP-RFA parasites were grown with 10 μM reduced GSH (green), 0.4 μM vitamin C (vitC,blue), or 5 mM N-acetyl-L-cysteine (NAC, orange) in the presence (circles) or absence (squares) of 10 μM TMP. Parasites were also grown in the presence (black circle) or absence (red square) of 10 μM TMP with DMSO as a vehicle control. Growth was monitored via flow cytometry. Data are fit to an exponential growth equation and are represented as mean ± SD. (n=3). Shown is a representative experiment from 3 repetitions, each sample done in triplicate.

Human		Matched Peptides	
Gene ID	Protein Description	3D7 iRBC	GURP-GFP
NCBI: 847	Catalase		5
NCBI: 5052	Peroxiredoxin 2		4
NCBI: 759	Carbonic Anhydrase 1		4
NCBI: 2040	Stomatin	7	7
NCBI: 2597	GAPDH	4	
NCBI: 287	Ankyrin	4	
NCBI: 6708	Spectrin	5	
P. falciparum		Matched Peptides	
Gene ID	Protein Description	3D7 iRBC	GURP-GFP
PF14_0598	GAPDH-glycolysis enyzme	8	9
PF10_0155	Enolase-glycolysis enzyme	7	
PF08_0054	Hsp70-molecular chaperone		4
PFB0923c	GURP	6	5

Table 1. Immunoprecipitation Mass Spectrometry Data. Identification of proteins pulled down with anti-GURP and anti-GFP antibodies from D10 and GURP-GFP expressing parasites, respectively. In red are proteins identified by both parallel immunoprecipitations. Results are one representative experiment out of two biological replicates.



Supplementary Figure 1. GURP is exported to the host RBC at various parasite stages. Deconvoluted Z-stack images of *P. falciparum* infected RBCs probed with anti-GURP antibody. Abbreviations: R, ring; S, schizont; G, gametocyte. Scale bar = 5 μm.



Supplementary Figure 2. Glut1 localization is unchanged.

Deconvoluted Z-stack images of *P. falciparum* iRBCs and uiRBCs stained with rabbit anti-Glut1 antibody. Scale bar = 5 μm.

Primer Name	Sequence (5' to 3')	Restriction Site	PCR Purpose	Comments
5'GURP	gggctgcagatgaatatgttttttttattc	PstI	Forward primer for GURP gene	Used in making GURP-pCR4-Topo
3'GURP	gggaccggttgcttcgggtttgtatattttaac	AgeI	Reverse primer for GURP gene	Used in making GURP-pCR4-Topo
5'AvrIIMut	ataccctacaaaggaaaaaagaatctactaggaaaaagattaggtttaatatc	N/A	Forward primer for synonymous mutation of internal GURP AvrII site	Used in making GURPmut-pCR4-Topo
3'AvrIIMut	gatattaaacctaatctttttcctagtagattctttttttcctttgtaggggtat	N/A	Reverse primer for synonymous mutation of internal GURP AvrII site	Used in making GURPmut-pCR4-Topo
5'GURPXhoI-GDB	cactatagaactcgagaatgattataatataccctacaaagg	XhoI	Forward primer for 561 bp homologous region in the 3' end of GURP	Used in making GURP-GDB
3'GURPAvrII-GDB	ctgcacctggcctagggtggtggtggtggtgtgcttcgggtttg	AvrII	Reverse primer for 561 bp homologous region in the 3' end of GURP	Used in making GURP-GDB
5'GURP-RFP	acgatttttttctcgagatgaatatgttttttttattc	XhoI	Forward primer for GURP cDNA	Used in making GURPcDNA-pCR4-TOPO
3'GURP-RFP	cctagggcccttagacaccattgcttcgggtttgtatattttaaccttggc	AvrII	Reverse primer for GURP cDNA	Used in making GURPcDNA-pCR4-TOPO
5'GURPpET	gggggatccgcggccgcaatgttaacagaagttatgatgcc	BamHI	Forward primer for GURP mature protein cDNA	Used in making GURPmat-pCR4-TOPO
3'GURPpET	ggggaattctgatgcttcgggtttgtatattttaaccttggc	EcoRI	Reverse primer for GURP cDNA	Used in making GURPmat-pCR4-TOPO
5' E55c-tyEOE	acgatttttttctcgagattccattttaaataaatacgaagg	XhoI	Forward primer for PFE0055c (Hsp40) gene	Used in making E55c-tyEOE
3' E55c-tyEOE	ctgcacctggcctaggatacgtattttccaaggtttcc	AvrII	Reverse primer for PFE0055c (Hsp40) gene	Used in making E55c-tyEOE

Supplementary Table 1. Sequence of primers used to generate constructs for this study.

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CHAPTER III

GURP C183 is Essential for Parasite Growth and Development

ABSTRACT

GURP acts by sequestering stomatin in double membrane vesicles in the red blood cell cytosol (RBC) of *P. falciparum* infected RBCs. In a recent study GURP was predicted to be palmitoylated, and prediction software suggested C183 as the site of modification. Palmitoylation is reversible post-translational modification known to play a role in membrane association of proteins and trafficking of proteins between membrane compartments (Smotrys and Linder, 2004). Here, we demonstrate that mutation of GURP C183 to serine traps vesicles in the parasitophorous vacuolar membrane (PVM). Parasites expressing the C183S mutation exhibit severe growth and morphological defects. Taken together, our data shows that C183 is involved in targeting GURP containing vesicles to the RBC cytosol and necessary for parasite growth in iRBCs.

INTRODUCTION

As aforementioned, one of the criteria we used when screening PlasmoDB for candidate proteins, was the presence of an odd number of cysteines. We chose this criterion because free cysteines are frequently observed in functionally important sites in proteins such as catalytic, regulatory, and cofactor binding sites (Marino and Gladyshev, 2010). Cysteines are also susceptible to a variety of post-translational modifications (PTMs) including S-nitrosylation (Hess *et al*., 2005), prenylation (Zhang and Casey, 1996), and palmitoylation (Tom and Martin, 2013). Interestingly, a recent study of the *P. falciparum* palmitoylome identified GURP as being palmitoylated (Jones *et al*., 2012).

Palmitoylation is the reversible addition of a 16-carbon saturated fatty acid to cysteine residues (Linder and Deschenes, 2007). Palmitoylation is involved in protein membrane trafficking, protein-protein interactions, and vesicular transport (Salaun *et al*., 2010). Given that GURP localizes to unique vesicles and was shown to be palmitoylated, we wanted to individually mutate each of GURP's three cysteines to determine what, if any, effect mutation would have on protein localization and parasite development during the intraerythrocytic cycle. We provide evidence here that mutation of GURP C183 to serine causes adverse effects on parasite growth and morphology and that the mutant protein is trapped in the parasitophorous vacuolar membrane (PVM).

RESULTS

Episomal expression of GURP-C48S or C138S has no effect on parasite growth

Parasites expressing wild-type, C48S, or C138S GURP protein were monitored in culture and growth was analyzed by flow cytometry. There was no visible growth difference between the parasites episomally expressing the wild-type or either GURP mutant protein. Interestingly, parasites transfected with the GURP-C183S plasmid did not come back after drug selection. All transfections were performed at the same time, which ruled out any technical issue. This led us to believe that constitutive episomal expression of GURP-C183S is toxic to the parasites.

Conditional episomal expression of GURP-C183S ellicits severe growth and morphological defects in parasites

To determine if expression of the GURP-C183S mutation is indeed toxic to parasites, we utilized the RFA tag that allows for conditional expression of your protein of interest (Muralidharan *et al.*, 2011; 2012). The tag contains the gene for green fluorescent protein (GFP) and a destabilized human dihydrofolate reductase domain (hDHFRdd). Parasites were transfected with episomal constructs expressing wild-type GURP or GURP harboring a mutation encoding C183S. Parasites were selected without trimethroprim (TMP) to keep the episomal GURP silent. To "turn on" the episomal wild-type or C183S mutant GURP, parasites were cultured in media with 10 μM TMP.

To determine if there was any growth defect, parasites were culture +/- TMP, collected over time, and analyzed by flow cytometry. Parasites carrying the GURP-C183S construct showed severe growth defects +TMP (Figure 3), compared to GURP wild-type expressing parasites. Blood smears (Figure 4), showed "empty space" in the parasite cytosol of C183S

parasites, while the wild-type parasites looked normal. Electron microscopy (Figure 4), showed complete separation of the parasitophorous vacuole membrane (PVM) and the parasite plasma membrane (PPM) in the C183S expressing parasites, +TMP. Neither the growth defect nor the morphological defect was seen in parasites expressing a synonymous mutation at C183, indicating that these phenotypes are not merely due to a nucleotide change. Although more severe in the C183S expressing parasites, the morphological changes are similar to those seen in the GURP knockdown parasites mentioned in the previous chapter. These data show that episomal expression of GURP-C183S induces a dominant negative effect, as these parasites retain expression of the native GURP protein. This indicates that C183 is necessary for GURP function.

C183 is necessary for targeting of GURP containing vesicles to the RBC cytosol

To determine if GURP C183S was still exported into the RBC, we took advantage of the GFP tag for live imaging of both mutant and wild-type GURP expressing constructs. While the wild-type GURP-RFA protein is exported into the RBC, most of GURP C183S-RFA remains in the parasitophorous vacuolar membrane (PVM) (Figure 5). Occasionally, one to two discreet foci are also seen in the mutant expressing parasites (Figure 5, white arrows). This suggests that C183 is necessary for targeting of GURP containing vesicles to the RBC cytosol. Those few vesicles that do make it to the RBC cytosol may contain more of the endogenous protein than the episomally expressed mutant protein, thus allowing proper targeting of the vesicles. This implies that GURP C183S acts as a dominant negative by trapping of the vesicles in the PVM, which prevents any endogenous protein from sequestering stomatin. Stomatin then remains free to bind to Glut1, mediating the switch from glucose to DHA transport, thus causing the mutant expressing parasites to exhibit a severe growth defect, presumably due to insufficient glucose.

DISCUSSION/CONCLUSIONS

Knockdown of GURP showed that GURP is important for parasite growth and glucose uptake during intraerythrocytic infection. Mutation of C183 causes severe growth and morphological defects, and trapping of GURP vesicles in the PVM. Recently, a study of the palmitoylome of *P. falciparum* (Jones *et al.*, 2012) indicated that GURP is palmitoylated and prediction software, such as CSS-Palm, suggests that C183 is the site of palmitoylation. Palmitoylation is a reversible modification that facilitates protein membrane interactions and trafficking, and it modulates protein-protein interactions and enzyme activity (Smotrys and Linder, 2004). Taken together, this suggests that palmitoylation is necessary for GURP containing vesicles to be targeted effectively to the host RBC cytosol.

It is worth noting that GURP (Jones *et al.*, 2012) and stomatin (Snyers *et al.*, 1999) are both palmitoylated. This begs the question of whether or not palmitoylation is involved in the GURP-stomatin interaction or in GURP's sequestration of stomatin in double membrane vesicles during *P. falciparum* RBC infection.

MATERIALS AND METHODS

DNA Sequences and Cloning

Total RNA was extracted from iRBCs as previously described (Kyes *et al.*, 2000). The GURP open reading frame was amplified by RT-PCR from *P. falciparum* total RNA using the primers listed in Table 1. The cDNA product was then cloned into the pCR4-TOPO vector (Invitrogen) and sequenced. An internal AvrII site was disrupted by introducing a synonymous mutation (GURPmut-TOPO) using the primers listed in Table 1 and the QuickChange XL Site-Directed Mutagenesis Kit (Stratagene). The cysteine mutants were introduced using the primers listed in Table 1 and the QuickChange XL Site-Directed Mutagenesis Kit (Stratagene) with the GURPmut-TOPO construct as the template DNA. GURP wild-type and cysteine mutant cDNA was then cloned into the episomal vectors using the In-Fusion technique (Clontech).

Cell Culture and Transfections

Plasmodium falciparum 3D7 parasites were cultured in RPMI medium supplemented with Albumax and transfected as described previously (Drew *et al.*2008; Russo *et al.*2009). For episomal expression, 3D7 parasites were transfected with pGURP-PM2GT (constitutive expression) or pGURP-GDB (conditional expression) which contains a human dehydrofolate reductase (PM2GT) or blasticidin deaminase (GDB) selection marker allowing selection for parasites maintaining the episome (Muralidharan *et al.*, 2012). Cultures underwent positive selection after 48h with either 10 nM WR (PM2GT) or 2.5 μg/mL BSD (GDB). For conditional episomal expression growth assays, GURP-GDB parasites were equally divided into two separate samples, with media containing 10 μM TMP (+TMP) added to one sample while drug free media was added to the other (-TMP). All cultures were plated in triplicate 1mL volumes for flow cytometry.

Flow Cytometry

Aliquots of parasite cultures (7 µL) were stained with 1.5 µg/mL Acridine Orange (Molecular Probes) in PBS (Muralidharan *et al.*, 2012). The fluorescence profiles of infected erythrocytes were analyzed by flow cytometry on a BD FACS Canto (BD Biosystems). The parasitemia data were fit to a standard exponential growth curve in the software package GraphPad Prism v 5.

Live Cell Imaging

Parasites expressing GURP wild-type or C183S-RFA were grown in the presence of 10 µM TMP for 48 hours. Samples were collected, stained with Hoescht DNA dye and imaged Axioscope microscope (Carl Zeiss).

Electron Microscopy

Parasites expressing GURP wild-type or C183S-RFA were grown in the presence of 10 µM TMP for 48 or 72 hours. Samples were purified by magnetic column (Ribaut *et al.*, 2007), washed in PBS, fixed in 1% osmium tetroxide (Polysciences Inc.) for 1 h. Samples were then rinsed extensively in distilled water before en bloc staining with 1%aqueous uranyl acetate (Ted Pella) for 1 h. Following several rinses in distilled water, samples were dehydrated in a graded series of ethanol solutions and embedded in Eponate 12 resin (Ted Pella). Sections of 95 nm were cut with a Leica Ultracut UCT ultramicrotome, stained with uranyl acetate and lead citrate, and viewed on a JEOL 1200 EX transmission electron microscope.

ACKNOWLEDGEMENTS

We would like to thank Wandy Beatty for performing the electron microscopy and Barb Vaupel for construction of the episomal plasmids used.

Figure 1. Schematic of GURP protein.

GURP has three cysteines and two C-terminal putative transmembrane domains, as shown. The PEXEL cleavage site is indicated by the dashed black line. Abbreviations: SP, signal peptide; P, PEXEL; TM, transmembrane domain.

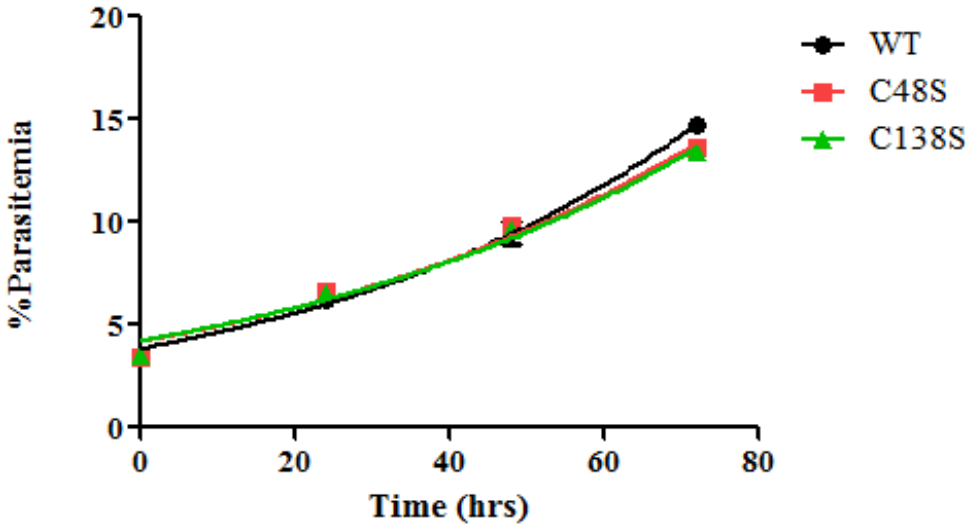

Figure 2. Constitutive expression of C48S and C138S had no growth effect.

GURP wild-type (black circle), GURP-C48S (pink square), and GURP-C138S (green triangle) expressing parasites were collected at indicated time points, stained with acridine orange, and anlayzed for parasitemia by flow cytometry. Data are fit to an exponential growth equation and are represented as mean ± S. E. M. (n = 3).

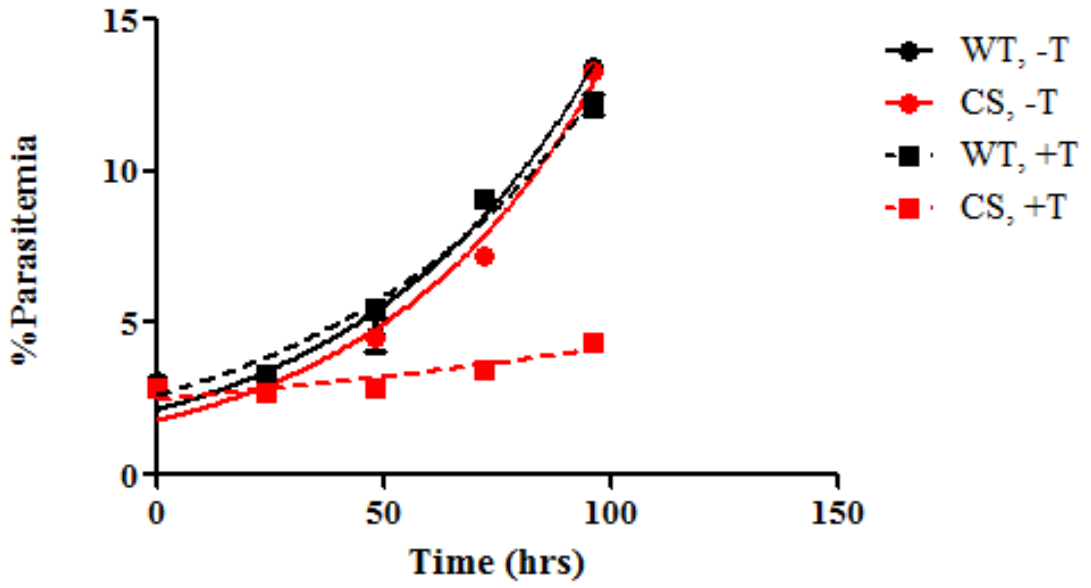

Figure 3. Expression of C183S causes a severe growth defect.

GURP wild-type (black) and GURP-C183S (red) expressing parasites were grown + (circles) or – (squares) 10 μm TMP (T), collected at indicated time points, stained with acridine orange, and analyzed for parasitemia by flow cytometry. Data are fit to an exponential growth equation and are represented as mean ± S. E. M. (n = 3).

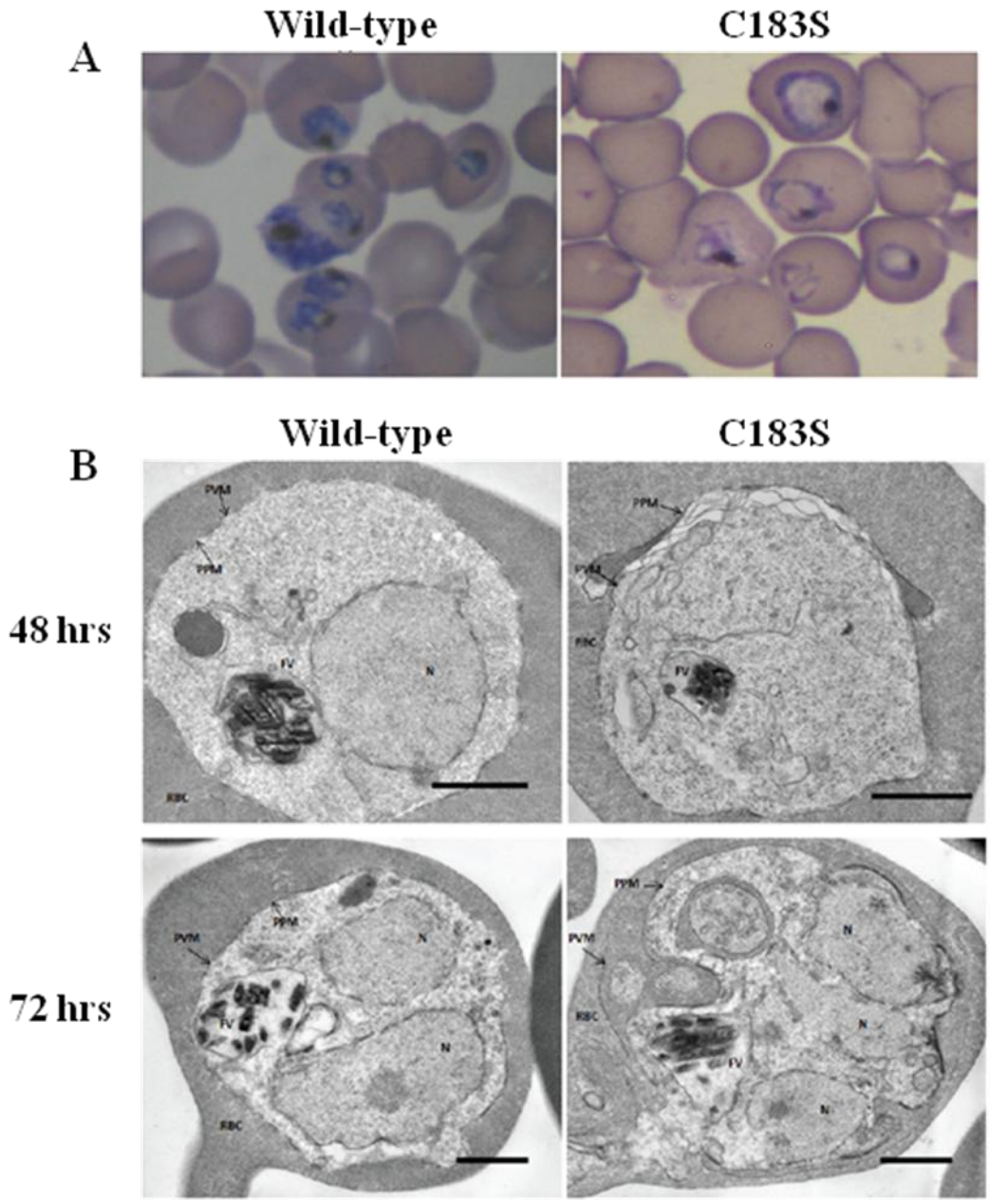

Figure 4. Expression of C183S causes severe morphological defects.

GURP wild-type and C183S expressing parasites were grown in 10 μM TMP. Samples were collected for (A) blood smear at 72 hours and (B) electron microscopy at 48 and 72 hours. Scale bar = 1 μm.

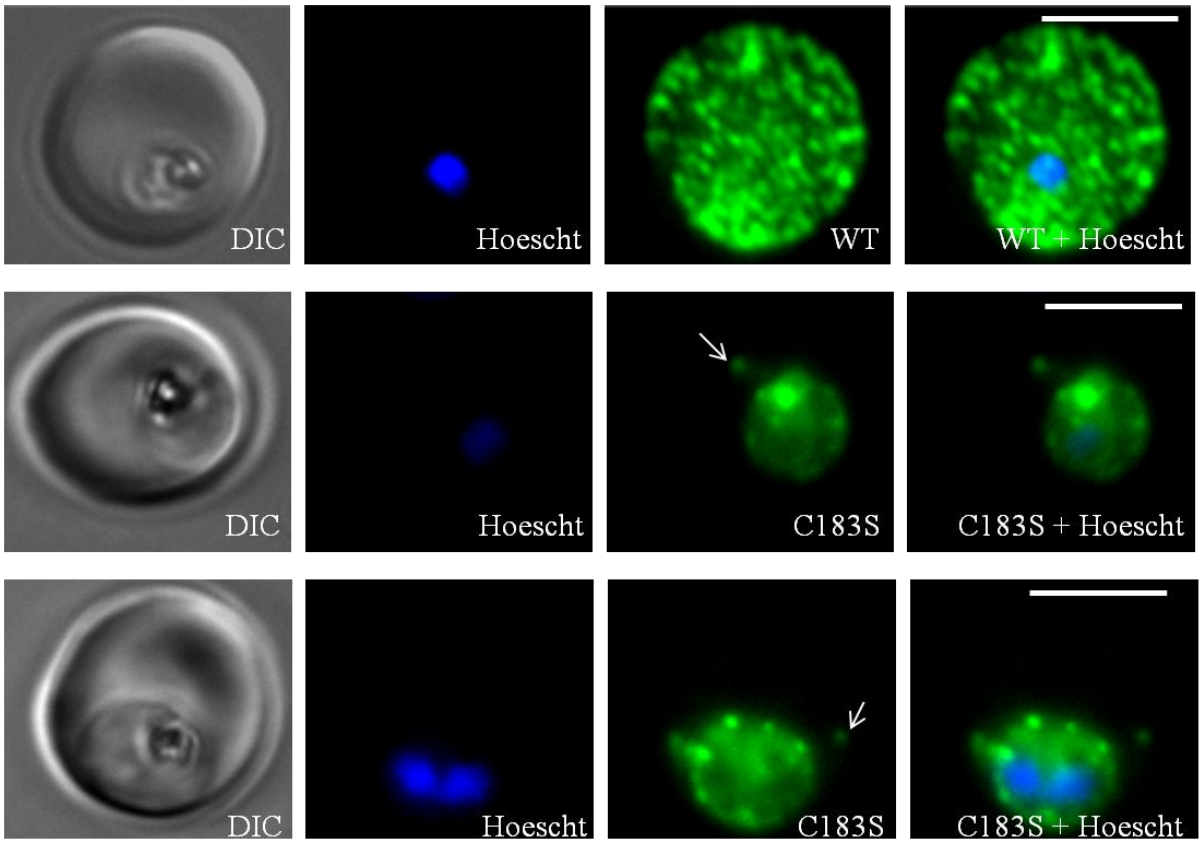

Figure 5. GURP C183 targets vesicles to the RBC cytosol.

GURP wild-type and C183S-RFA expressing parasites were grown in 10 μM TMP for 48 hours. Samples were collected, stained with Hoescht DNA dye and analyzed by live microscopy. Scale bar = 5 μm.

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CHAPTER IV

Conclusions and Future Directions

INTRODUCTION

In this body of work, we have shown that the exported *P. falciparum* protein, GURP, interacts with and sequesters the host protein stomatin, in double membrane vesicles in the RBC cytosol. Stomatin binds to several channels and transporters, including Glut1 (Rungaldier *et al.*, 2013). Overexpression of stomatin depresses Glut1 glucose transport (Zhang *et al.*, 2001). Sequestration of stomatin by GURP presumably prevents stomatin binding to Glut, which we hypothesized would cause an increase in glucose transport. However, functional knockdown of GURP shows that glucose metabolism is decreased, not transport, as evidenced by lack of 2-DOG accumulation via phosphorylation. This suggests that GURP is possibly upregulating host hexokinase (HK) function, as HK catalyzes phosphorylation of glucose to G6P. Interestingly, hexokinase activity is downregulated during erythrocyte maturation (Shinohara *et al.*, 1985) but upregulated in *P. falciparum* iRBCs (Roth, 1987; Roth *et al.*, 1988). Measuring RBC HK activity in GURP knockdown parasites will determine if GURP plays a role in regulating host HK during intraerythrocytic infection.

GURP was predicted to be palmitoylated in a recent study of the *P. falciparum* palmitome (Jones *et al.*, 2012). Palmitoylation is reversible post-translational modification known to play a role in membrane association of proteins and trafficking of proteins between membrane compartments (Smotrys and Linder, 2004). Individual mutation of each of GURP's three cysteines showed that C183 is essential for parasite growth and development. C183 is also necessary for targeting of the novel, GURP containing, double membrane vesicles to the RBC cytosol, as expression of a C183S mutant traps GURP in the parasitophorous vacuole membrane (PVM). These data suggest that palmitoylation plays a role in trafficking of GURP to vesicles in the RBC cytosol or of GURP- containing vesicles to the RBC cytosol, and that GURPs

localization in the cytosol is necessary for parasite growth and development during intraerythrocytic infection.

GURP's essentiality during RBC infection, and its uniqueness to *P. falciparum*, suggests it would be an appropriate drug target. Delineating the residues necessary for GURP-stomatin and stomatin-Glut1 interactions will provide further understanding of the mechanism of the parasites sequestration of stomatin, and of how stomatin acts to regulate Glut1 function. Determining the contents of GURP containing vesicles may lead to identification of additional parasite proteins that are necessary for parasite survival and open the doors for exploration of the RBC cytosol to discover other novel parasite-derived organelles.

INHIBITION OF GURP AS POTENTIAL THERAPEUTIC TREATMENT FOR *P. FALCIPARUM* MALARIA

Malaria infections have classically been attributed to one of five species of the human malaria parasites, *Plasmodium falciparum, P. vivax, P. ovale, P. malariae* (Richie, 1988), and P. knowlesi (White, 2008). In 2010, there were 219 million malaria cases, and 660,000 deaths from malaria; *Plasmodium falciparum* accounted for 91% of the overall deaths (World Health Organization (WHO), 2012). Treatment of malaria has been hampered by the emergence of drug resistant parasites.

Quinine was the first drug used to treat malaria (Dobson, 2001) and continues to be used today (Achan *et al.*, 2011), although cases of quinine resistance have been reported in some areas (Dedet *et al.*, 1988). In Thailand, mefloquine was introduced as first-line treatment in 1984 and significant resistance developed within 6 years (Price *et al.*, 2004). Chloroquine (CQ), a derivative of quinine, was the most widely-used drug from the early 1950s to the 1990s. After ten years of use, mutations within *P. falciparum* that conferred resistance to CQ arose and since then, CQ-resistant mutations have been spreading quickly throughout most endemic areas (Bloland, 2001; Mita *et al.*, 2009).

Sulfadoxine-pyrimethamine (SP), a combination of two drugs, replaced CQ. However, resistance to SP evolved rapidly and now occurs at high frequency in major endemic regions (Laxminarayan, 2004). Since 2001, the WHO has recommended artemisinin-based combination therapy (ACT), a combined regimen of artemisinin and a longer-acting partner drug, as the treatment choice for *falciparum* malaria (WHO, 2010). The recent emergence of *P. falciparum* strains with increased resistance to artemisinins in Southeast Asia, raised the possibility that these drugs might already be obsolete (Noedl *et al.*, 2008; Dondorp *et al.*, 2009; Wongsrichanalai

and Sibley, 2013). Therefore, there is a dire need to develop new drug treatments against malaria.

GURP is unique to *P. falciparum* and has no similar proteins in humans. These characteristics, coupled with its essentiality to parasite development during RBC infection, make GURP an attractive drug target. Delineating the interface of GURP-stomatin complex would be the first step toward designing an inhibitor to repress GURP function. This can be done by crystallizing the GURP-stomatin complex and identifying the residues involved in the GURP-stomatin interaction. Obtaining purified GURP and stomatin proteins presents a challenge as stomatin has known membrane domains and GURP has putative membrane domains. Strategies successfully employed to purify other membrane proteins (Smith, 2011) could be applied to GURP and stomatin purification.

UNDERSTANDING THE GURP-STOMATIN INTERACTION

Although we know that GURP interacts with stomatin from IP and mass spec data, we do not know what residues are necessary for this interaction. Attempts to express GFP-tagged N- and C-terminal truncations of GURP in parasites have been unsuccessful; only the full-length GURP construct was expressed as evidenced by fluorescence microscopy. Ideally, crystal structures of the GURP-stomatin complex would elucidate the interaction interface; however, efforts to obtain soluble GURP protein have been unsuccessful and as stomatin is a membrane protein as well, similar difficulties can arise.

Structure predictions of GURP (Figure 1) using the Robetta (robetta.bakerlab.org) and COACH (Yang *et al.*, 2013) servers are similar. The Robetta prediction has a more open conformation while the COACH prediction is more closed. The structure prediction of GURP (COACH prediction) in complex with mouse stomatin (Figure 2) was made using GRAMM-X simulation (vakser.bioinformatics.ku.edu). This prediction has residues in putative transmembrane domain 2 of GURP interacting with stomatin. Although the predictions are a place to start, actual experimental data is necessary. Optimizing methods to express and purify soluble protein are crucial for determining the mechanics behind the GURP-stomatin interaction, and eventually designing inhibitors for the GURP-stomatin interface.

We have shown that GURP acts by sequestering stomatin in vesicles in the RBC cytosol and that C183 is necessary for targeting these vesicles to the cytosol. This implies that stomatin is delivered/recruited to the vesicles and subsequently bound by GURP to prevent its interaction with Glut1 (Figure 3). Determining the mechanism of stomatin's delivery/recruitment to these vesicles would present another potential target for malaria treatment.

MEASURING HEXOKINASE ACTIVITY IN *P. falciparum* INFECTED RED BLOOD CELLS

The glucose uptake assays performed with GURP knockdown parasites showed that metabolism (phosphorylation), not transport, of glucose was decreased upon GURP knockdown. Glucose is converted to glucose-6-phosphate by the glycolytic enzyme hexokinase. Hexokinase activity can be measured by colorimetric assay where glucose is converted to glucose-6-phosphate (G6P) by hexokinase. The G6P is then oxidized by glucose-6-phosphate dehydrogenase (G6PDH) to form NADH, which reduces a colorless probe to a colored product with absorbance at 450 nm (Abcam). Absorbance of unknown samples is then compared to an NADH standard curve to determine enzyme activity.

As the parasite has its own active HK (Roth, 1987; Tjhin *et al*., 2013) that localizes to the parasite plasma membrane (Olafsson and Certa, 1994), steps will be taken to ensure that only the red blood cell HK activity is measured. P. falciparum iRBCs will be lysed with tetanolysin O (tetO), which selectively permeabilizes the red blood cell membrane, leaving the parasitophorous vacuole membrane intact (Lopez-Estrano *et al.*, 2003). Supernatants from tetO lysis will be used in assays measuring HK activity, to determine if GURP knockdown affects red blood cell HK during infection.

VERIFYING THE GURP-PfGAPDH INTERACTION

Another interesting protein that was identified in the GURP co-immunoprecipitation assays was *P. falciparum* glyceraldehyde-3-phosphate dehydrogenase (PfGAPDH). GAPDH is classically characterized as a glycolytic enzyme, catalyzing the oxidative phosphorylation of glyceraldehyde-3-phosphate into 1,3-bisphosphoglycerate. PfGAPDH is expressed throughout the intraerythrocytic life cycle and displays enzymatic activity with NAD^+ (Daubenberger *et al.*, 2000). PfGAPDH has an active site cysteine residue that is conserved (Daubenberger *et al.*, 2000) and found to be essential for enzyme activity in human GAPDH (Molina y Vedia *et al.*, 1992; McDonald and Moss, 1993).

Recently, independent labs have identified diverse biological properties of mammalian GAPDH protein (Sirover, 1999), including roles in membrane transport and membrane fusion (Lopez Vinals *et al.*, 1987; Han *et al.*, 1998; Hessler *et al.*, 1998; de Arcuri et a., 1999; Bressi *et al.*, 2001; Glaser *et al.*, 2002). The N-terminal domain of PfGADPH binds to microsomal membranes in response to the GTPase Rab 2 (Daubenberger *et al.*, 2003), suggesting that PfGAPDH also has functions outside of the glycolytic pathway. If the PfGAPDH-GURP interaction is real, PfGAPDH could be functioning in stomatin delivery/recruitment to GURP containing vesicles. Reciprocal immunoprecipitation assays, immunofluorescence and immuno-EM colocalization assays will need to be performed to verify that GURP and PfGAPDH are interacting.

IDENTIFYING NEW PARASITE DERIVED ORGANELLES IN iRBCS

Maurer's clefts (Sam-Yellowe *et al.*, 2004), J-dots (Kulzer *et al.*, 2010; 2012), and K-dots (Kats *et al.*, 2014) are all parasite derived organelles or vesicles in the red blood cell cytosol. We have shown that GURP does not localize to Maurer's clefts or J-dots. Whether GURP localizes to K-dots remains to be seen.

Determining the contents of the GURP vesicles may lead to the discovery of additional parasite proteins involved in the GURP-stomatin sequestration or in other mechanisms the parasite uses to hijack the RBC for its purposes. Differential velocity centrifugation of lysed iRBCs, followed by affinity purification with anti-GURP antibody, may be able to isolate the GURP vesicles for protein identification by mass spectrometry. Lysates from uninfected red blood cells will be included to control for non-specific hits. Proteins that are unique to the iRBC sample will be episomally expressed with a fluorescent tag that can be used IP and microscopy to verify localization.

The fact that GURP and FIKK4.2 (K-dots) do not localize to these known parasite derived structures makes one wonder what other organelles are present in the RBC cytosol. Determining the localization of more exported proteins of unknown function may shed light on what other structures the parasite has manufactured to assist in commandeering the host RBC.

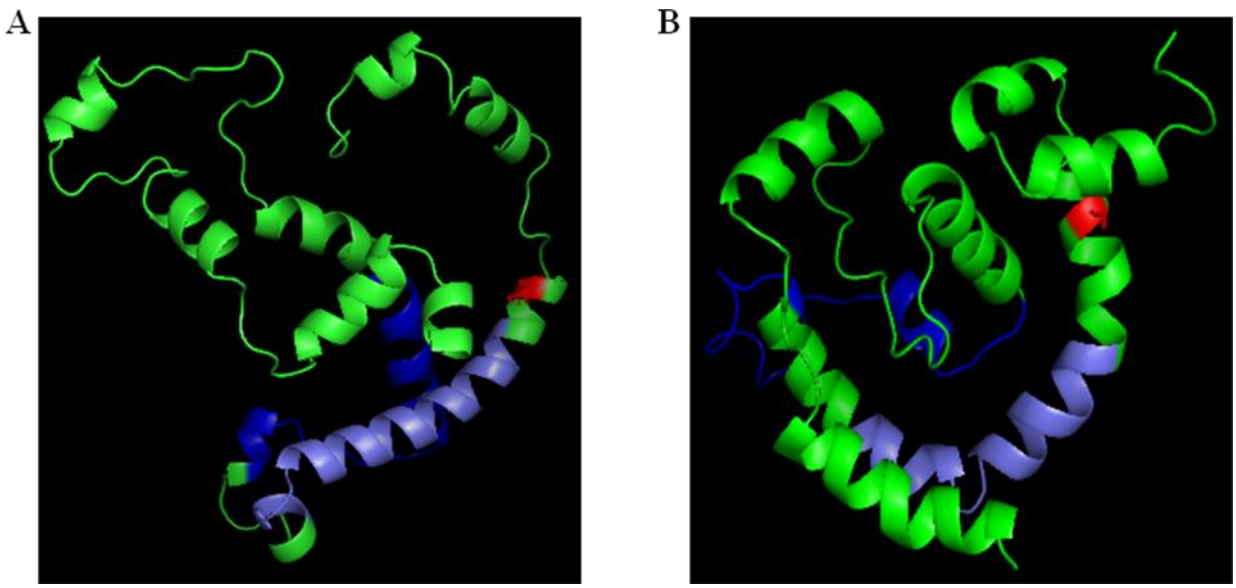

Figure 1. GURP structure predictions.

Structure predictions of mature GURP courtesy of **(A)** Robetta (http://robetta.bakerlab.org) and **(B)** COACH (http://zhanglab.ccmb.med.umich.edu/COACH) servers. TM1 (dark blue), TM2 (light blue), and C183 (red) are highlighted. Abbreviation: TM, putative transmembrane domain. Images courtesy of the PyMOL Molecular Graphics System, Version 1.3 Schrödinger, LLC.

Figure 2. GURP-stomatin complex structure prediction.

GURP-stomatin complex prediction using GURP structure from COACH (Figure 1B) and mouse stomatin (light blue) structure solved by Brand *et al.*, 2012. GURP TM2 is involved in the contact with stomatin. Structure courtesy of GRAMM-X (vakser.bioinformatics.ku.edu). Image courtesy of the PyMOL Molecular Graphics System, Version 1.3 Schrödinger, LLC.

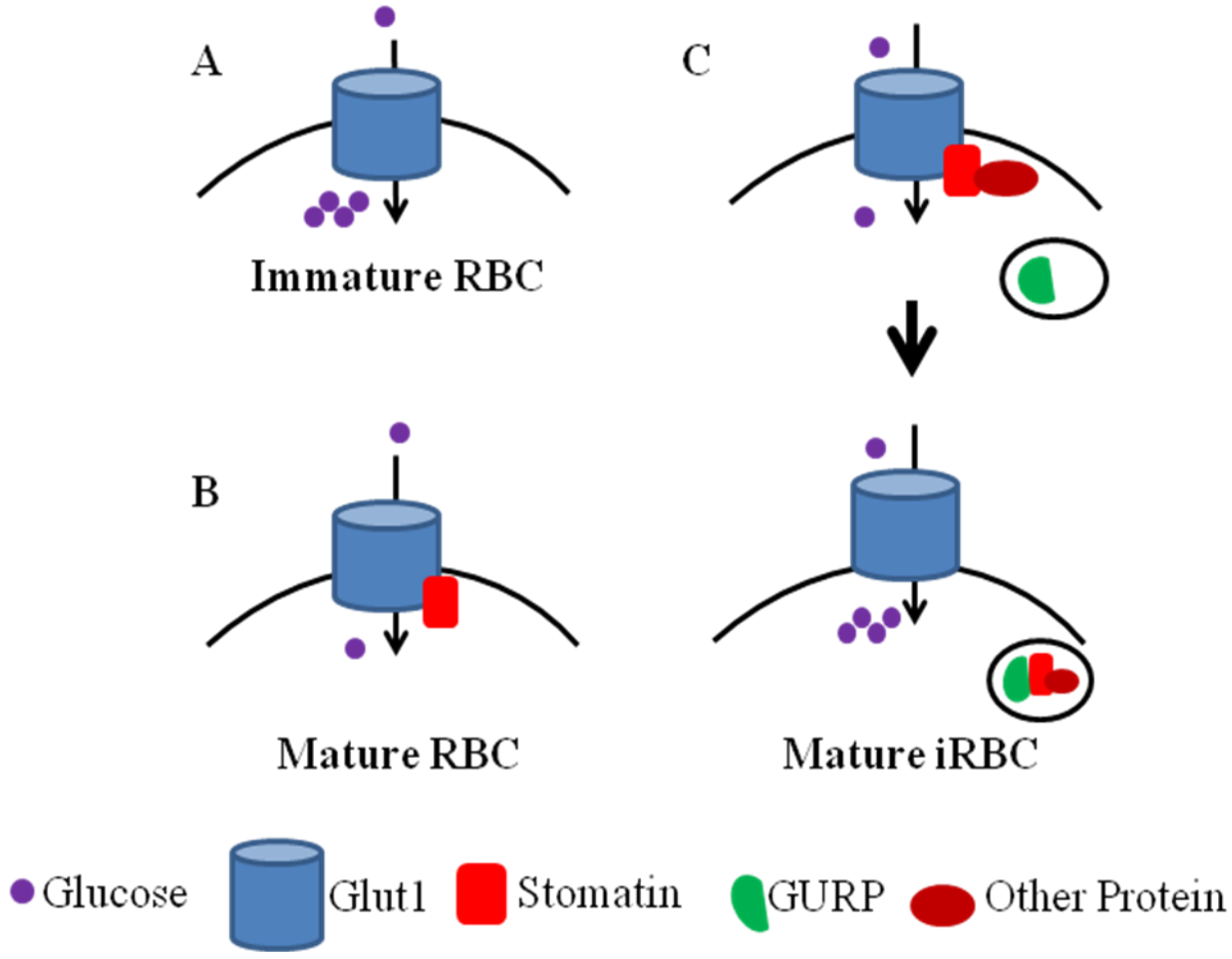

Figure 3. Schematic representation of the proposed mechanism of stomatin sequestration in iRBCs.

(A) Glut1 transports glucose at high levels in immature RBCs. **(B)** In mature RBCs, when stomatin is highly expressed, it depresses Glut1 glucose transport. **(C)** In mature *P. falciparum* iRBCs, stomatin is sequestered by GURP in vesicles in the RBC cytosol, preventing its interaction with Glut1, thus increasing glucose uptake. Presumably, another protein delivers/recruits stomatin to the vesicles where it is bound by GURP.

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APPENDIX I

Half and Half: Processing of a Putative Parasite SMC Protein

INTRODUCTION

In addition to screening PlasmoDB for candidate hypothetical proteins to study, we also performed an in vitro screen with Cy5- maleimide to label proteins with free sulfhydryl groups. Maleimide is a chemical compound that readily reacts with the thiolate group found on free cysteines to form a stable carbon-sulfur bond. The Cy5 dye allows visualization of the labeled proteins after separation by 2D gel analysis (Figure 1). The protein that we chose from this screen was PFF0835w (PFF).

PFF is a hypothetical, 53 kDa protein with three cysteines, eight predicted phosphorylation sites and a domain similar to the structural maintenance of chromosomes (SMC) family of proteins (Figure 2). Mutational analysis of the *Bacillus subtilis* SMC protein has shown that cysteines are necessary for dimerization of SMC subunits and their interaction with DNA (Hirano and Hirano, 2002). Furthermore, a stretch of amino acids in the PFF SMC domain was found to be antigenic against sera from adult donors from Burkina Faso, Tanzania, and Colombia (Villard *et al.*, 2007) and children from Kenya (Agak *et al.*, 2008).

SMC proteins are ATPases that play important roles in sister chromatid cohesion, chromosome condensation, sex-chromosome dosage compensation, and DNA recombination and repair (Kim *et al.*, 2002). They are conserved in bacteria, archaea, and eukaryotes (Losada *et al.*2005). The *P. falciparum* SMC proteins are found to be highly conserved amongst *Plasmodium* species which suggests that they may play an important role as a component of the chromosomal maintenance complex and are probably indispensable for the parasite (Gangwar *et al.*2009).

RESULTS

PFF mRNA is transcribed throughout *P. falciparum* RBC development

Since SMC proteins are involved with chromosomal maintenance, we hypothesized that the protein would be expressed at all parasite developmental stages. As we did not yet have the gene tagged or an antibody to the protein, we used RT-PCR to determine mRNA expression levels throughout the intraerythrocytic cycle. PFF mRNA was expressed at each stage of the parasite RBC life cycle (Figure 3A), which is in contrast to most *Plasmodium* genes that exhibit a single transcriptional peak (Bozdech *et al.*, 2003; Le Roch *et al.*, 2003).

PFF is cleaved, localizes to parasite cytosol

Given that all other SMC proteins have been found to localize to the chromosomes, we hypothesized that PFF would localize to the parasite nucleus. To test this, we introduced a GFP tag at the 3' end of the PFF endogenous locus by single crossover homologous recombination. However, although a Southern blot showed that we achieved integration, we were unable to visualize any GFP signal by Western blot, live microscopy, or Immunofluorescence assays (IFA). This suggested that tagging the C-terminus of the protein interfered with its function, therefore, we episomally introduced a copy of PFF with an N-terminal hemagglutinin (HA) tag.

We were able to visualize the tagged protein by both Western blot (Figure 3B) and IFA (Figure 3C) with anti-HA antibody. Interestingly, the Western blot showed two bands: one at approximately 54 kDa and a smaller band at 25 kDa, indicating cleavage of the protein. Assuming this cleavage occurs at the C-terminus would explain why we failed to see any signal with the C-terminal GFP-tagged protein.

IFA showed that PFF localized throughout the parasite cytosol and in some discreet foci (Figure 3C). Some staining did seem to surround the nucleus; however, there was no evidence of nuclear localization.

DISCUSSION/CONCLUSIONS

Maleimide staining of *P. falciparum* iRBC lysates identified several hypothetical proteins. We chose to pursue PFF0835w because of its SMC domain and the importance of SMC proteins across biological systems. The 53 kDa size of PFF is unconventional for SMC proteins, which are normally 130-145 kDa. Western blot of parasites expressing an N-terminal HA tagged PFF revealed that it is further cleaved almost in half, resulting in a 25 kDa protein. The size of the HA-tagged protein suggests that PFF is cleaved approximately at residue 211, which is within the SMC domain. This is not to rule out that the proteolysis could occur during cell lysis.

As SMC proteins are involved with chromatin segregation and partitioning, one would expect a nuclear localization. IFA showed that PFF is distributed throughout the parasite cytosol with some localization to discreet foci. Taken together, these data inspire doubt that PFF is an actual SMC protein. However, the N-terminal tag could interfere with localization, although the small size of the HA-tag makes interference less plausible. Nevertheless, the constitutive mRNA transcription profile, the fact that PFF is processed, has several phosphorylation sites, and possesses a lone cysteine after cleavage, are all characteristics that warrant further study of PFF0835w.

The HA-tagged PFF parasite line can be used for co-immunoprecipitation experiments to identify any interacting partners and potentially provide insight on PFF's actual function in *P. falciparum*. HA-tagged protein can be purified from iRBCs and analyzed by mass spectrometry to determine the cleavage site.

MATERIALS AND METHODS

RNA isolation and RT-PCR

For RNA extraction, iRBC pellets were lysed in 1 ml prewarmed (37°C) Trizol LS reagent (Gibco Invitrogen) and then stored at −80°C in screw top cryovials. RNA was extracted as described previously (Kyes *et al.*, 200), using DNase I digestion (Invitrogen) to remove contaminating DNA. RNA was then reverse transcribed (SuperScript III One-Step RT-PCR, Invitrogen) using 5'-GGTGGTGGTGCTCGAGATGACTGAAGCTGAAAATATAAAAATCG-3' forward primer and 5'-GAATTAATTCGGATCCAGCCTTAACTTTATTTGCCTTGG-3' reverse primer.

DNA sequences and cloning

The *PFF* open reading frame was amplified by RT-PCR (SuperScript III One-Step RT-PCR, Invitrogen) from *P. falciparum* total RNA using 5'-GGGGGATCCGCGGCCGCAATGACTGAAGCTGAAAATATAAAAATC-3' forward primer and 5'- GGGGTCGACGAATTCTGAAGCCTTAACTTTATTTGCCTTGGTTCC-3' reverse primer. The cDNA was cloned into the pCR4-TOPO vector (Invitrogen), and sequenced. For episomal expression, PFF cDNA was cloned into the PM2GT vector (Muralidharan *et al.*, 2012) using the In-Fusion cloning system (Clontech) and primers to introduce a 5'-HA tag (5'-ACGATTTTTTCTCGAGGGGCCTAGGATGTACCCATACGATGTTCCAGATTACGCTATGACTGAAGCTGAAAATATAAAAATCGAAAAACC-3') and a stop codon (5'-TAACTCGACGCGGCCGCTTAAGCCTTAACTTTATTTGCCTTGGTTCCTTTGTTGTGATG-3') before the GFP tag in the vector.

Cell Culture and Transfections

Plasmodium falciparum 3D7 parasites were cultured in RPMI medium supplemented with Albumax and transfected as described previously (Drew *et al.*, 2008; Russo *et al.*, 2009). Parasites transfected with pPFF-5'HA underwent positive selection after 48h with 10 nM WR. WR was re-added with every change of culture medium. Greater than 90% of parasites displayed fluorescence when stained with anti-HA antibody and, indicating they were carrying the plasmid.

Western Blot

Samples separated by SDS-PAGE were transferred to nitrocellulose membrane (Fisher) and incubated for 1 hour in blocking buffer (LICOR Biosciences). HA-tagged PFF was detected with primary rat anti-HA (1:1000) (Roche) antibody and secondary IRDye 800CW (1:10000) conjugated donkey anti-rat (LICOR Biosciences) antibody. The western blot images were processed and analyzed using the Odyssey infrared imaging system software (LICOR Biosciences).

Indirect Immunofluorescence Assay (IFA)

HA-tagged PFF expressing parasites were fixed, permeabilized (Ponpuak *et al.*, 2007) and incubated with rabbit anti-HA antibody (1:50) (Sigma) and Alexa Fluor™555 donkey anti-rabbit antibody (1:1000). Cells were pre-layered on PEI-treated coverslips and mounted in Prolong Gold antifade with DAPI (Molecular Probes). Cells were observed on an Axioscope Microscope (Carl Zeiss Microimaging).

ACKNOWLEDGEMENTS

We would like to thank Barb Vaupel for construction of the PM2GT vector and Mark Drew for assistance with 2D-gel procedure.

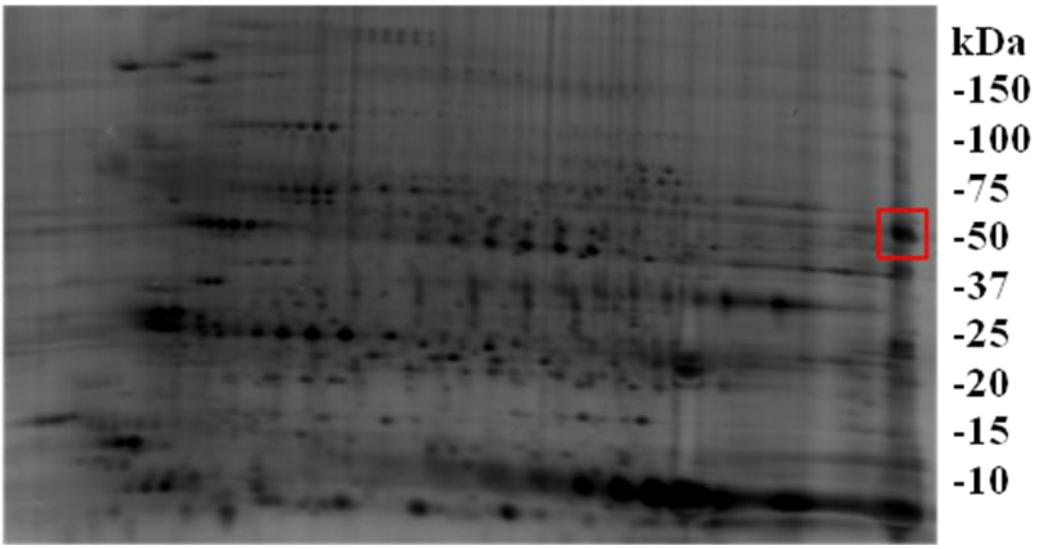

Figure 1. 2D-gel analysis of Cy5-maleimide labeled parasite extracts.

Infected RBCs were incubated with Cy5-maleimide to label free sulfhydryl groups. Samples were first separated by isoelectric focusing, followed by separation by SDS-PAGE. The spot corresponding to PFF0835w is indicated by the red square.

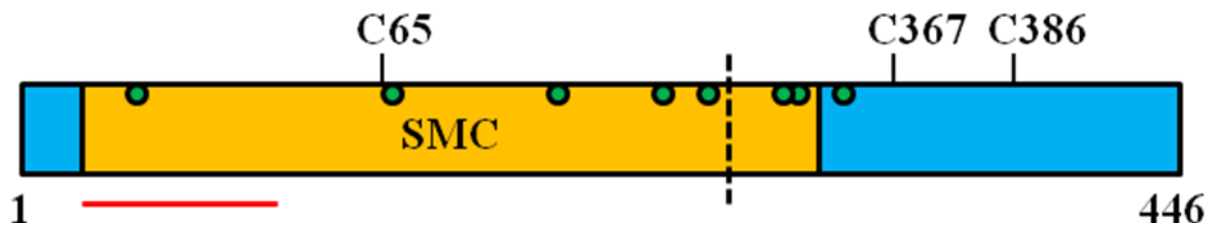

Figure 2. Schematic of PFF0835w protein.

PFF has three cysteines as shown. Residues 12-248 contain the SMC domain (orange). Predicted phosphorylation sites are shown as green dots. Residues 12-47 were recognized as antigenic (red bar). The putative cleavage site is indicated by the dashed black line.

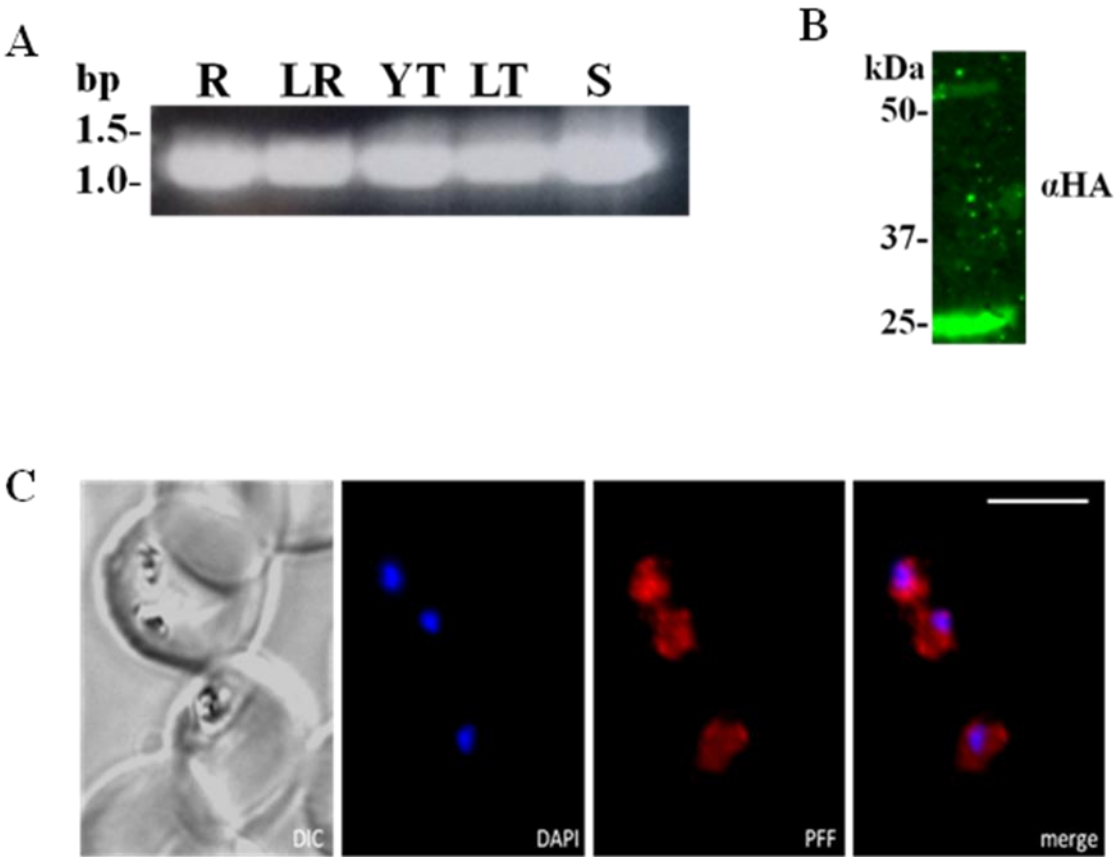

Figure 3. PFF0835w is expressed, cleaved, and localizes to the parasite cytosol.

(A) Agarose gel of RT-PCR done with total RNA from parasite RBC stages. (B) Western blot and (C) IFA of PFF-HA expressing parasites. Scale bar = 5 μm. Abbreviations: R, ring; LR, late ring; YT, young trophozoite; LT, late trophozoite; S, schizont.

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APPENDIX II

Plasmepsin V Licenses Plasmodium Proteins for Export into the Host Erythrocyte

Vol 463|4 February 2010|doi:10.1038/nature08726

ARTICLES

Plasmepsin V licenses *Plasmodium* proteins for export into the host erythrocyte

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During their intraerythrocytic development, malaria parasites export hundreds of proteins to remodel their host cell. Nutrient acquisition, cytoadherence and antigenic variation are among the key virulence functions effected by this erythrocyte takeover. Proteins destined for export are synthesized in the endoplasmic reticulum (ER) and cleaved at a conserved (PEXEL) motif, which allows translocation into the host cell via an ATP-driven translocon called the PTEX complex. We report that plasmepsin V, an ER aspartic protease with distant homology to the mammalian processing enzyme BACE, recognizes the PEXEL motif and cleaves it at the correct site. This enzyme is essential for parasite viability and ER residence is essential for its function. We propose that plasmepsin V is the PEXEL protease and is an attractive enzyme for antimalarial drug development.

The human malaria parasite *Plasmodium falciparum* exports an estimated 200–300 proteins into the host erythrocyte[1,2]. In doing so, the parasite remodels the cytoskeleton and plasma membrane to create cytoadherence knobs, nutrient permeation pathways and altered erythrocyte mechanical stability[3,4]. Export of these effectors is dependent on a *Plasmodium* export element or PEXEL sequence, RxLxE/Q/D[5,6]. Proteins destined for export are cleaved after the conserved PEXEL leucine in the ER and mutation of the R or L residues attenuates cleavage and export[7,8]. Plasmepsin V (PMV) is an aspartic protease that has distant homology to mammalian BACE or beta-secretase[9], an enzyme involved in the processing of amyloid precursor protein[10]. Both have a carboxy-terminal extension that contains a hydrophobic membrane anchor sequence. An amino-terminal aspartic protease 'pro-domain' remains unprocessed in PMV[9]. PMV is expressed in intraerythrocytic *P. falciparum* parasites and has orthologues in other *Plasmodium* species. *Phytophthora infestans*, the potato blight pathogen that has a similar export system[11], has a homologous sequence in the database (PITG_02623.1). PMV has been localized to the ER[9] and is therefore a candidate to be the PEXEL processing protease.

Role of the transmembrane domain in PMV localization

PMV lacks a classical ER retention signal. To identify the element responsible for localization and to assess the importance of ER residence for PMV function, we made sequential C-terminal truncation mutants (Fig. 1a). Single crossover homologous recombination into the endogenous locus was performed, introducing a green florescence protein (GFP) tag after a full-length C terminus or in place of C-terminal sequence (Fig. 1b). Full-length PMV–GFP integrants (clone DC6) and integrants with deletion of the C terminus downstream of the membrane-spanning segment (clone EF2) had no phenotype and retained ER targeting (Fig. 1c–e and Supplementary Fig. 2), whereas deletions involving the membrane anchor were lethal (Fig. 1b). Fusion of the transmembrane region but not other portions of PMV was sufficient to target a reporter to the ER (Fig. 1f). Thus the transmembrane sequence is important for ER localization and probably for cellular activity on its substrates as well.

PMV essentiality

We further assessed essentiality of the PMV gene for intraerythrocytic parasites by using an allelic replacement approach[12]. An integration vector in which the first catalytic aspartate was modified by synonymous or non-synonymous mutation was transfected into parasites (Fig. 2a) and recombinants were obtained (Fig. 2b, c). Crossover into the endogenous PMV gene proved possible only when the aspartate codon was preserved (replacement via the synonymous mutation vector or downstream crossover bypassing the mutation with the non-synonymous mutation vector) (Fig. 2d). Non-synonymous alteration of the active site codon could not be achieved in four separate transfection experiments. These results support the notion that PMV has an essential function in the cell.

Dominant-negative PMV phenotype

To investigate PMV function, we episomally expressed two GFP-tagged versions of PMV, one wild type and the other containing a D to A mutation in the active site aspartate 108 to render expressed protein catalytically dead. Both versions localized to the ER but the mutant had threefold reduced signal by immunofluorescence (Fig. 3a–c) and by western blot (Fig. 3d). Mutant enzyme-expressing parasites were frequently seen encased in erythrocyte ghosts (Fig. 3e), indicating impaired host cell homeostasis. Indeed the mutant PMV-expressing culture grew more slowly than the wild-type PMV-expressing culture (Fig. 3f). Occasional cells expressing the mutant construct had intense signal similar to wild type; in such cases two parasites could be seen in a single erythrocyte, one of which was not fluorescent and had presumably lost or downregulated the plasmid (Fig. 3g). Our interpretation of this result is that the plasmid-free parasite can export proteins normally into the shared red blood cell, overcoming the effect that the mutant PMV has on the neighbouring parasite. To explore this further, we assessed processing of the exported histidine-rich protein II (HRPII) in the PMV-transfected cells (Fig. 3h, i). Unprocessed HRPII was barely detectable in wild-type PMV-expressing parasites. In contrast, unprocessed HRPII accumulated in mutated PMV-expressing parasites. Plasmepsin II and DPAP1, non-PEXEL containing proteins that use the secretory pathway but are then internalized instead of exported[13,14], were

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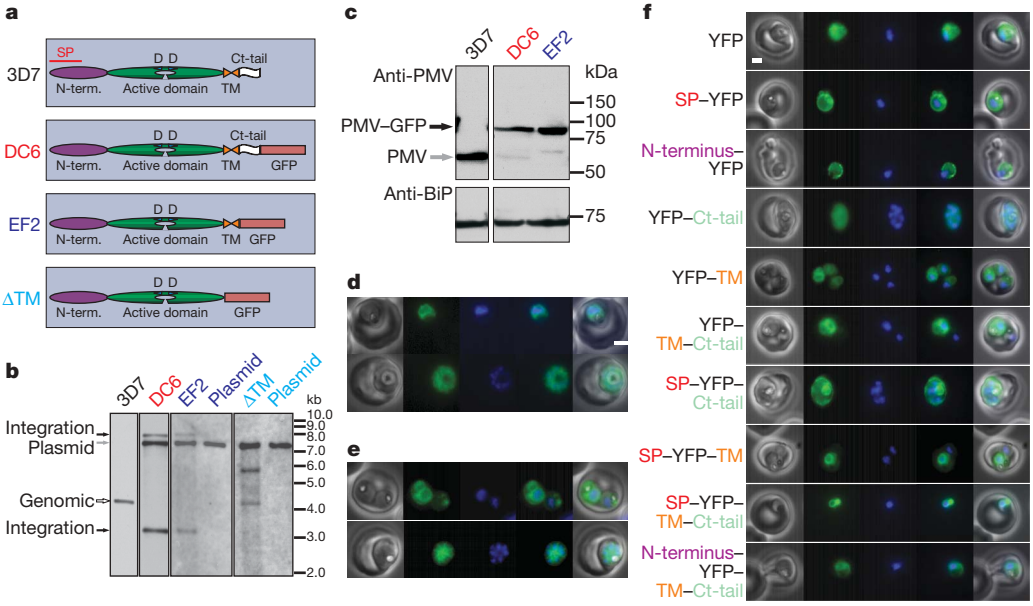

Figure 1 | The transmembrane region of plasmepsin V confers ER localization. **a**, Schematic of PMV and C-terminal integrants. SP, signal peptide; N-term., N-terminal domain; Ct, C-terminal; TM, transmembrane region. **b**, Southern blots of full-length (DC6) or C-terminal tail deletion (EF2) integrant clones and transmembrane region deletion transfectants (ΔTM). Parental strain (3D7) and plasmids are shown for reference. **c**, Western blot using anti-PMV antibody. BiP served as loading control. **d**, **e**, Live fluorescence images of DC6 and EF2, respectively. Left to right: phase, GFP, DAPI, fluorescence merge and total merge. Scale bar, 2 μm. **f**, Localization of PMV fragments (defined in **a**) fused to yellow fluorescent protein (YFP). Expression was episomal, using the HSP86 promoter. Panel order as in **d** and **e**.

processed normally (Fig. 3i). Export was assessed by immunofluorescence (Fig. 3j, k). The levels of host erythrocyte HRPII and another exported protein, RESA (ring-infected erythrocyte surface antigen), were diminished in the mutated PMV-expressing parasitized erythrocytes by 30–50%. These data indicate that episomal expression of catalytically dead PMV has a dominant-negative effect on parasite growth and on protein export. A survey of PEXEL gene essentiality estimated that about one-fourth are required for intraerythrocytic parasite growth[15]. Thus, perhaps 50–75 exported proteins are essential. Some will be required at near wild-type levels for optimal growth, whereas others will tolerate more drastic reduction without consequence. The 30–50% reduction in protein export is therefore about what would be expected, given the growth phenotype seen (Fig. 3f).

Enzyme activity and specificity

To assess enzyme activity, PMV–GFP was detergent-solubilized from recombinant clone DC6 (see Fig. 1a, c) and enzyme was isolated using anti-GFP antibody. The enzyme was able to cleave a fluorogenic decapeptide based on the PEXEL motif from the exported HRPII (Fig. 4a, b). Pull-downs from the parental strain (3D7) with untagged PMV had no activity. When anti-PMV antibody was used for enzyme isolation, both tagged (DC6) and untagged (3D7) enzyme could be isolated and were active. A second PEXEL peptide based on PfEMP2 was also cleaved by isolated enzyme (Fig. 4b). Mutation of P1 Leu or P3 Arg abolishes export of PEXEL proteins[10]. Peptides with either of these residues changed to Ala were refractory to cleavage (Fig. 4b), confirming specificity of the enzyme for the

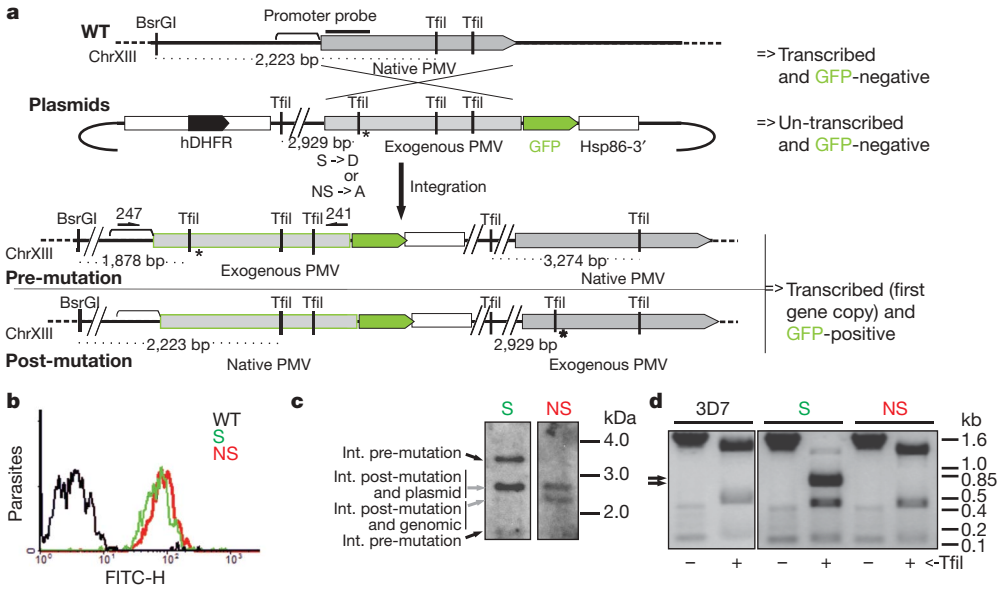

Figure 2 | Plasmepsin V is essential for intraerythrocytic parasite viability. **a**, Active site allelic replacement scheme. Integration vectors possessing a synonymous (S) or non-synonymous (NS) mutation in the Asp 108 codon and a new TfiI restriction site just upstream were transfected into parental strain 3D7. Possible outcomes for upstream crossover (pre-mutation) and downstream crossover (post-mutation) are shown. Integrants were selected and assessed by PCR. Primers used for amplification (241 and 247) are marked. **b**, Flow cytometry of parasites. Parasites were assessed for GFP expression, an indication of integration at the endogenous locus. Parental strain 3D7 (WT) is shown as control. **c**, Southern blot of transfected parasite pools. Int., integration. At left are expected positions of possible products of the BsrGI/TfiI digest mapped in **c**. **d**, Screening of integrants by PCR and restriction digest. Arrows indicate a doublet (predicted 787 and 748 bp) resulting from restriction fragmentation of product from recombinants that have crossed over upstream of the active site codon.

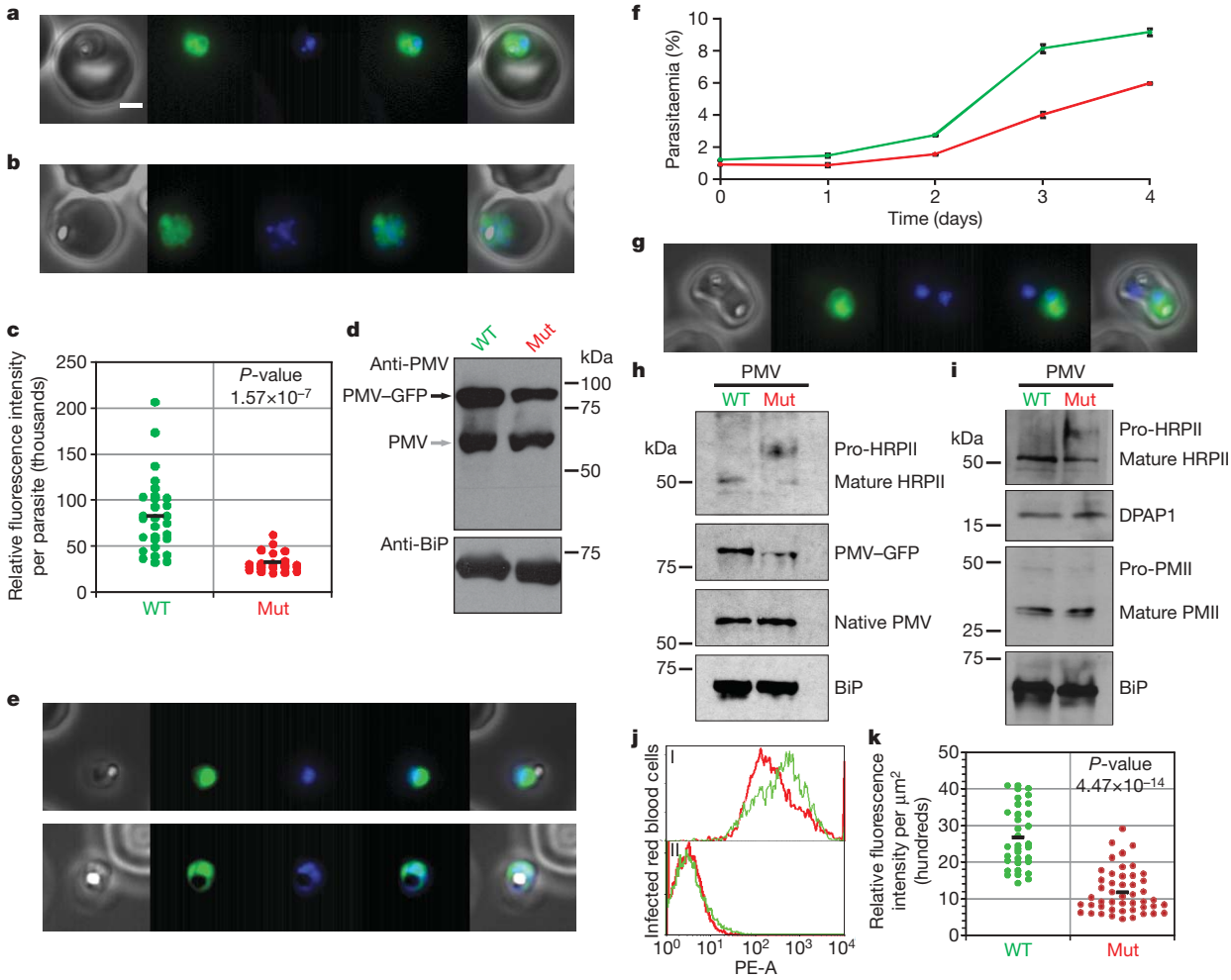

Figure 3 | Episomal expression of wild-type and catalytic mutant PMV. **a**, **b**, Immunofluorescence of wild-type and 108 D to A (mutant) PMV expressing lines, respectively. Left to right: phase, GFP, DAPI, merge. Bar, 2 μm. **c**, Fluorescence intensity of 52 transfected early trophozoites was measured. Mean relative fluorescence units were 82,559 and 32,059 for wild-type and mutant-transfected parasites, respectively. **d**, Western blot. BiP serves as a loading control. **e**, Mutant PMV-expressing parasites encased in erythrocyte ghosts. **f**, Growth curves. Asynchronous cultures episomally expressing wild-type (green) or mutant (red) PMV were monitored by flow cytometry for four days in triplicate (error bars indicate standard deviation). Mutant growth rate is reduced by 49.2 ± 1.6%. **g**, Double-infected erythrocyte containing a parasite expressing high levels of mutant PMV. **h**, **i**, Western blots of HRPII processing in parasites episomally expressing mutant or wild-type PMV. **h**: top, anti-HRPII; middle panels, anti-PMV; bottom, anti-BiP. Contrast and brightness enhanced slightly over the entire panel to bring out features seen on the original film. **i**: top, anti-HRPII; middle panels, antibodies against non-exported secretory proteins dipeptidyl peptidase I (DPAP1) and plasmepsin II (PMII); bottom, anti-BiP. **j**, Flow cytometry. Wild-type (green) and mutant (red) PMV-expressing parasites were fixed, treated with tetanolysin (5 min, 20 units ml^{-1}) to selectively permeabilize the erythrocyte compartment and exported RESA was quantified by flow cytometry with specific antibody. Control without primary antibody is shown below. Results are representative of four experiments. **k**, HRPII staining was inconsistent by the fixation procedure used in **j**, so HRPII erythrocyte fluorescence intensity (80 cells) was quantified. Mean relative fluorescence intensity per μm^2 was 2,671 and 1,179 for wild-type and mutant, respectively.

PEXEL motif. Activity was seen between pH 5 and 7 (Fig. 4c). The pH of the mammalian ER has been measured to be 7.1 (ref. 16). Although we do not know the pH of the *Plasmodium* ER and cannot reproduce the cellular ionic environment in our *in vitro* assays, the measured activity is consistent with an ER function. Activity of the PMV active site mutant enzyme was undetectable (Fig. 4d). This result shows that PMV itself is the active protease, not an associated protein. Others have obtained active recombinant enzyme from *E. coli*[17], another indication that PMV is the protease in question. PMV, but not control preparations, cleaved a full-length PEXEL-containing proprotein (Fig. 4e).

To confirm cleavage specificity, the products of the HRPII PEXEL peptide incubation with isolated wild-type PMV enzyme were fractionated by reverse phase HPLC (Fig. 5a) and analysed by mass spectrometry (Fig. 5b). The fragments generated corresponded to proteolysis after the leucine that is the *in vivo* processing site. Similar results were obtained using the PfEMP2 peptide (Fig. 5c, d).

PMV interactions

We have shown that PMV is an essential ER protease in *Plasmodium falciparum*. Residence in the ER is necessary for its function, as deletion of the C-terminal tail had no effect on location or parasite viability whereas deletion of the transmembrane (TM) region rendered parasites non-viable (Fig. 1). Our data suggest that PMV is the enzyme that processes PEXEL-containing proteins to send them on their way for export into the host cell. It is not clear how PEXEL-containing proteins are recognized by the translocon in the parasitophorous vacuole[18] when most of the PEXEL has been cleaved off by PMV in the ER. It is conceivable that the propeptide stays associated with the mature polypeptide during transport, but we favour a model (Supplementary Fig. 1) in which chaperones associate with PMV in the ER. On PEXEL cleavage, these chaperones receive the protein destined for export, usher it through the secretory pathway and then thread it through the translocon channel. In support of this, PMV pull-downs (Fig. 6) consistently identified an ER-resident HSP70 and

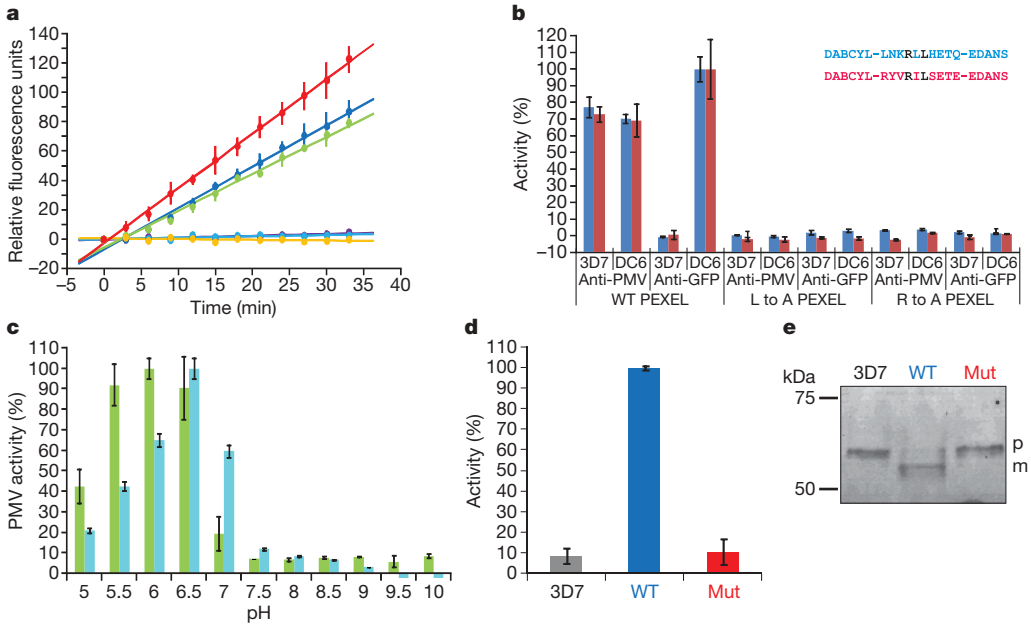

Figure 4 | Plasmepsin V activity. **a**, Substrate cleavage. PMV was isolated from parental (3D7) and PMV–GFP fusion (DC6) clones using anti-GFP (yellow and red, respectively) or anti-PMV (blue and green, respectively). A similar isolation without antibody (protein A) served as a control for non-specific binding (purple and cyan, respectively). Purified enzyme was incubated at pH 6.5 for 35 min with fluorogenic peptide (AnaSpec) corresponding to the PEXEL motif for HRPII (DABCYL-LNKRLLHETQ-EDANS). Error bars indicate standard deviation of three activity curves. **b**, PMV specificity for PEXEL motif. Processing of fluorogenic peptides containing the PEXEL motifs of HRPII (blue) or PfEMP2 (red, DABCYL-RYVRILSETE-EDANS) are shown. Left, wild-type peptide (WT); middle, L to A mutant peptide; right, R to A mutant peptide. Inset: peptide sequences with mutated residues in black. Error bars as in **a**. **c**, pH dependence in Tris-malate buffer. Two separate experiments are shown in different colours. Error bars as in **a**. **d**, Activity of active-site mutant PMV compared to wild type. PMV–GFP was isolated from 3D7 (untransfected mock isolation), wild-type- or mutant-PMV–GFP-transfected parasites using anti-GFP for purification. Activity on HRPII–PEXEL peptide is normalized to PMV protein content. Error bars as in **a**. **e**, Cleavage of pro-HRPII by isolated enzyme from **d**. p, pro-form; m, mature protein.

HSP101 (a key translocon component[18]) as associated proteins. Much remains to be done to define the PMV-chaperone relationship but it is certainly plausible that chaperones could act in a complex or relay to shepherd proteins from ER to translocon for export.

Enzyme inhibition

In vitro enzyme activity was partially inhibited by high micromolar concentrations of HIV protease inhibitors or pepstatin A (Supplementary Fig. 3a) but not by other classes of inhibitors. We tested a panel of protease inhibitors for ability to block processing of the PEXEL-containing exported protein HRPII but have not yet found a good inhibitor. BACE inhibitors had minimal effect, perhaps not surprising given the evolutionary distance between the two orthologues. Only HIV protease inhibitors had any effect and the blockade was partial (Supplementary Fig. 3b, c). Action on PMV is unlikely to be their primary effect because they kill cultured parasites in the single digit micromolar range[19,20], whereas effects on protein export and on isolated PMV were observed at 50–200 micromolar concentrations.

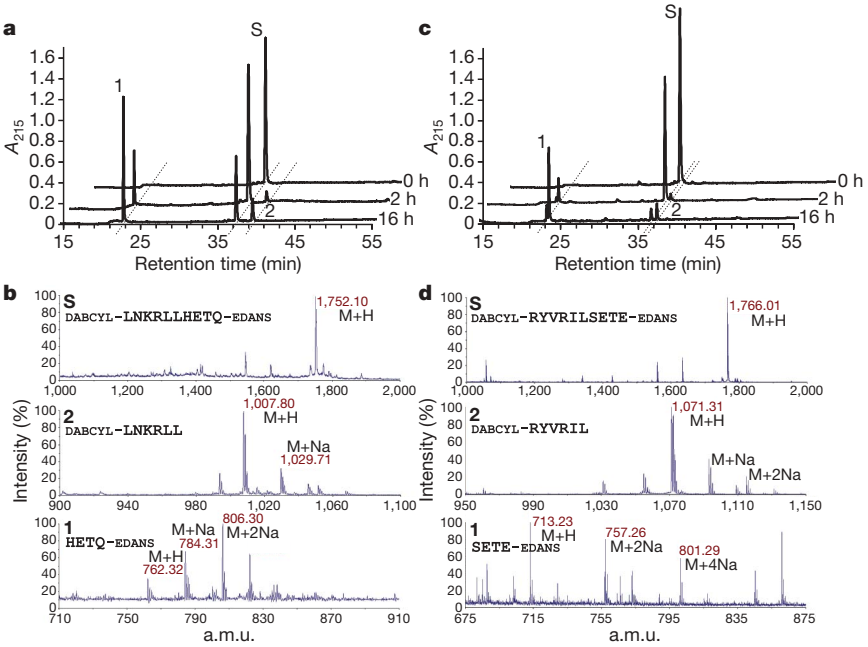

Figure 5 | Analysis of substrate cleavage. **a**, **c**, PMV was incubated with HRPII (**a**) or PfEMP2 (**c**) PEXEL peptides (DABCYL-LNKRLLHETQ-EDANS and DABCYL-RYVRILSETE-EDANS, respectively). Cleavage products were separated on a C18 column by reverse-phase HPLC. Back to front: incubation for 0, 2 and 16 h. S, substrate peak. **b**, **d**, Isolated products and substrates from **a** and **c** were analysed by MALDI-TOF mass spectrometry. Ion peaks and sodium adducts are labelled. HRPII peptide product masses: calculated 762.51 and 1,008.25; detected 762.32 and 1,007.80. PfEMP2 peptide product masses: calculated 1,071.30 and 713.72; detected 1,071.31 and 713.23. Ions corresponding to alternative peptide bond cleavage were not detected. a.m.u., atomic mass unit.

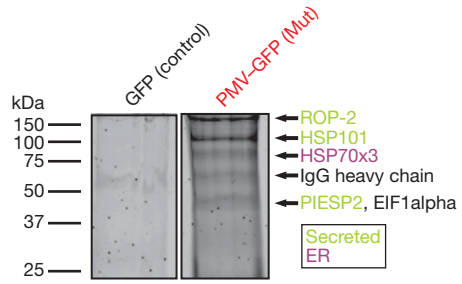

Figure 6 | Proteomic analysis of PMV-associated proteins. Coomassie-stained gel of an anti-GFP pull-down for the episomal GFP-expressing control strain (GFP) and the mutant PMV–GFP-expressing strain (Mut). Bands were excised, trypsinized and analysed by MS–MS. The same analysis was also performed on the wild-type PMV–GFP strain, and on the parental 3D7 strain but using anti-PMV antibody. Proteins for which at least four peptides were identified from the anti-PMV pull-down and three or more peptides from at least one of the two anti-GFP pull-downs of episomal PMV-expressing parasites are shown. All proteins identified are tabulated in Supplementary Table 1. None of the proteins were detected in the GFP control pull-down.

We propose that plasmepsin V is the PEXEL protease. This enzyme recognizes a simple RxL motif on secretory proteins destined for export into the host erythrocyte. Because PMV cleaves the PEXEL sequence away from the mature protein, the simplest conclusion is that PMV is primarily responsible for the specificity of export. An xE/Q/D dipeptide at the N terminus of mature exported proteins is also important for export though not for the cleavage itself[8]. Perhaps this polar residue comprises a secondary recognition element that interacts with the chaperone that will bring the protein to the translocon for export. It is very likely that the physical association of an escort system with PMV is needed to transfer the license for export. PMV seems then to be the gatekeeper for protein export. If potent inhibitors can be found, blocking the entire parasite virulence and intracellular survival program with one stroke will be a promising new strategy for combating this organism.

METHODS SUMMARY

Techniques for parasite culture, 3′-end integrations and truncations and their analysis, allelic replacement, site-directed mutagenesis, fluorescence imaging, parasite extraction and western blotting, as well as flow cytometry growth monitoring, have been described previously[12,21]. Parasite fluorescence intensity was measured blinded on random fields using Velocity 4 software (Improvision). For enzyme isolation, 50 ml of parasite culture at 2% haematocrit, 10% parasitaemia was harvested and parasites freed by saponin treatment as described[12]. Cells were solubilized for 30 min in 0.5% Triton X-100 in PBS buffer and incubated with anti-GFP (3E6; Invitrogen) or anti-PMV[9] antibodies for 1 h at 4 °C. Immune complexes were collected using protein A-Sepharose, washed extensively with PBS and isolated enzyme released in the activity buffer (see below) with 2 mM DTT. PMV enzyme activity assays were performed by incubation at 37 °C in 50 mM Tris-malate, pH 6.5, 50 mM NaCl, 0.05% Triton X-100 and reaction progress monitored on a Bio-Rad fluorimeter. Activity against pro-HRPII (ref. 22) was assessed after 16 h incubation by SDS–PAGE/anti-HRPII western blot. C18 reverse-phase liquid chromatography/MALDI mass spectrometry was performed as reported[23]. For pull-downs, enzyme was isolated as earlier except that solubilization was performed in RIPA buffer[21]. Immunoprecipitates were fractionated by SDS–PAGE and gel slices were analysed by MS–MS after trypsinization[24]. Primers for allelic replacement diagnosis were 241: AATTCCTAGGAGAAACTTTTAAGAAGATATTTTCTTTTTCTCTATATTC and 247: AATTCCTAGGAGAAACTTTTAAGAAGATATTTTCTTTTTCTCTATATTC. PMV sequences for 3′ fusion constructs were full-length, nucleotides 280 to 1770; tail deletion, nucleotides 280 to 1721; transmembrane region deletion, nucleotides 280 to 1623. Mutagenesis oligonucleotides for the allelic replacements: S, CGCAAAGAATTTCTTTGATTCTAGACACAGGTTCATCTTCGTTAAGTTTCCCGTG; NS, CGCAAAGAATTTCTTTGATTCTAGCGACAGGTTCATCTTCGTTAAGTTTCCCGTG.

Received 30 July; accepted 7 December 2009.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

Acknowledgements This work was supported by NIH grant AI-047798. We thank A. Cowman, B. Crabb and S. Lindquist for suggestions, J. Adams and ATCC (MR4) for BiP antibody, D. Taylor for HRPII antibody, R. Anders for RESA antibody, A. Miller and M. Ndonwi for HRPII protein, P. Hruz for HIV protease inhibitors, J. Tang and S. Romeo for BACE inhibitors, W. Beatty for fluorescence analysis, S. Beverley for fluorimeter access, B. Vaupel for technical assistance and J. Turk for mass spectrometer access. Proteomics analysis was carried out at the 'Fingerprints' Proteomics Facility, College of Life Sciences, University of Dundee.

Author Contributions I.R. designed and executed most of the experiments and wrote the paper; S.B., V.M., T.B. and A.O. designed and executed experiments; D.E.G. designed experiments and wrote the paper.

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