Part 1

Protein trafficking machinery in the malaria parasite: Molecular Characterization of Plasmodium falciparum homologues of p24 protein
Introduction
Introduction

The insurmountable success of *Plasmodium falciparum* in inhabiting terminally differentiated denucleated host cells is assertively due to the in-time and targeted trafficking of parasite effectors to their correct destinations. Apart from exporting proteins beyond its confines, at least a dozen distinct protein destinations are known to exist within the parasite precincts. Trafficking of proteins to distinct destinations in any eukaryotic cell requires a highly efficient and organized protein secretion and sorting machinery. In the absence of sorting determinants in the sequence of cargo proteins, the adaptor proteins play a crucial role in trafficking of cargoes to their correct destinations. In yeast and mammals, p24/gp25L/emp24/Erp family of putative adaptors has been identified, which is thought to play a crucial role in protein sorting and trafficking.

The aim of this study was to understand the trafficking and sorting machinery deployed by the parasite whilst ER export, for accurate protein targeting. To investigate this, the role of p24 orthologs in *P. falciparum* was explored by examining their localization and trafficking within the secretory pathway. These proteins were transcribed in the late stages and contained the conserved motifs required for interaction with the coat components of trafficking machinery. To ascertain their localization within the secretory pathway, transgenic parasites expressing full length p24 proteins appended with GFP tag at their C-terminus were generated. Analyses of transfectants showed that Pfp24.1 in general behaved as other known p24 proteins and recycled between ER and Golgi, whereas Pfp24.2 was localized in the PV lumen following its egress from ER. The accurate trafficking of these proteins was shown to be crucially dependent on the motifs in their short C-terminal tail. The redistribution of these proteins to ER in the presence of BFA, established these proteins as fundamental constituents of classical vesicle mediated trafficking machinery. Ultra structural analyses strongly supported their dynamic localization throughout the endomembrane system in tubo-vesicular structures and interestingly revealed a continuum channel, which apparently extended from ER to PV. These observations provided insights into the presence of explicit protein sorting
machinery in *P. falciparum*, dependent on specific adaptors. The study showed that the initial steps of secretory pathway in *P. falciparum* were mediated by protein machinery that is evolutionarily conserved across the species. Further, this data suggested the role of Pfp24 proteins in ferrying MSP1 and RHopH3 to PV, and depicted a possible mechanism by which early transcribed Rhoptry proteins were enriched in PV sub-domains during early biogenesis of Rhoptries. Overall, this study enlightens the unexplored realms of secretory pathway within the parasite, and gives an opportunity to unravel the machinery involved in initial protein sorting of essential parasite effectors.
Review of Literature
Malaria is caused by protozoans of the genus *Plasmodium*, which belong to the phylum Apicomplexa that also contains other human and animal pathogens such as *Toxoplasma*, *Cryptosporidium*, *Eimeria*, *Babesia* and *Theileria*. Apicomplexa are characterized by the presence of a unique organelle called apicoplast and an apical complex composed of three unique secretory organelles (rhoptries, micronemes and dense granules), juxtaposed to the nucleus. The genus *Plasmodium* contains more than 100 species of which four infect humans: *Plasmodium falciparum*, *P. vivax*, *P. ovale* and *P. malariae*. Other species like *P. knowlesi* and *P. cynomolgi* infect monkeys whereas *P. yoelii*, *P. berghei*, *P. chabaudi*, *P. vinckei*, cause infection in rodents. Recently, *P. knowlesi* has been recognized as the fifth *Plasmodium* species causing malaria in humans (1).

The large, exact numbers that delineate the immense and persistent burden of malaria, have become a familiar part of discussions in the global public health forum: 3 billion people at risk of infection in 109 countries and territories, and around 250 million cases annually, leading to approximately 1 million deaths [Fig 1]. In 2004, *P. falciparum* was among the leading causes of death worldwide, which makes malaria as the world's largest parasitic disease, responsible for killing more people than any other communicable disease except tuberculosis (2). Malaria is commonly-associated with poverty and poses major hindrance to the economic development worldwide.

Clinical manifestations of severe malaria include cerebral malaria, severe anaemia, hypoglycaemia, renal failure, non-cardiac pulmonary edema, and respiratory failure (2). Once in the blood, the processes associated with the multiplication of the parasite in the red blood cells (RBCs), are responsible for most of the clinical symptoms of malaria and its associated morbidity and mortality.

No vaccine is yet available and resistance to most antimalarial drugs has already been documented in many parts of the world. Research is mainly focused on *P. falciparum* because of its clinical importance. In addition, an *in-vitro* culturing system is available (3) and the genome has been sequenced for the culture strain 3D7 (www.plasmodb.org).
nuclear genome of *P. falciparum* (3D7) contains 22.8 million base pairs distributed on 14 chromosomes (4). About 5300 coding genes have been identified, of which more than 60% have no assigned function as they lack homology to any known protein based on their primary sequence.

![Fig. 1. Estimated incidence of malaria per 1000 population, 2006 (adapted from World Malaria report 2008, World Health Organization).](image)

**Historical perspective**

Malaria has infected humans for over 50,000 years, and may have been a human pathogen for the entire history of our species (5). The ancient Egyptians recognized and described malaria in detail. Findings from 30 million year old fossils of mosquito suggest that the vector for malaria was present well before the earliest history. Indeed, close relatives of the human malaria parasites remain common in chimpanzees, our closest relatives (6). References to the unique periodic fevers of malaria are found throughout recorded history, beginning 2700 BC in China (7).
The term malaria originated from Medieval Italian: *mala aria* — "bad air"; and the disease was formerly called *ague* or *marsh fever*, due to its association with swamps and marshy areas around ancient Rome (8). From their origin in Africa, malaria parasites were possibly brought to the New World by early trans-Pacific voyager, and also by consignment of slaves bound for the Spanish colonies, and this trend of imported malaria still continues till date. So by early 1800’s, malaria was worldwide in its geographical distribution.

Hippocrates was the first to describe the manifestation of the disease, and related them to the time of year and place where the patients lived. Before this, the supernatural powers were blamed for causing the disease. However, scientific studies on malaria made their first significant advance in 1880, when a French army doctor working in Algeria named Charles Louis Alphonse Laveran, observed parasites inside the red blood cells of people suffering from malaria. He therefore proposed that malaria was caused by protozoan and for the first time a protozoan was identified as the causative agent of any disease. For this and later discoveries, he was awarded the 1907 Nobel Prize for Physiology or Medicine. This protozoan was named as *Plasmodium* by the Italian scientists Ettore Marchiafava and Angelo Celli. An year later, Carlos Finlay, a Cuban doctor treating patients with yellow fever in Havana, first suggested that mosquitoes were transmitting disease to and from humans, but it was Britain's Sir Ronald Ross working in India who finally proved in 1898 that malaria is transmitted by mosquitoes. He did this by showing that certain mosquito species transmitted malaria to birds and isolated malaria parasites from the salivary glands of mosquitoes that had fed on infected birds. For this work Ross received the 1902 Nobel Prize in Medicine. First recorded effective treatment of malaria dates back to 1600, when bitter bark of cinchona tree was used by the native Peruvian Indians to control malaria. Later, Jesuits introduced this practice to Europe during the 1640s, where it was rapidly accepted (9). However, it was not until 1820, when the active ingredient was extracted from the bark and named as "Quinine", by the French chemists Pierre Joseph Pelletier and Jean Bienaime Caventou. By 1885, asexual development and
reproduction by multiple fission and correlation between the beginning of fever and rupture of infected erythrocyte was shown by Golgi. Although the blood stage and mosquito stages of the malaria life cycle were established in the 19th and early 20th centuries, it was not until the 1980s that the latent liver form (hypnozoites) of the parasite was observed (10). The discovery of this latent form of the parasite finally explained why people who appeared to be cured of malaria still relapse years after the parasite had disappeared from their bloodstreams.

Life cycle of Plasmodium

Malaria parasites have a complex life cycle [Fig. 2]. Infection in humans begins, when an infected female Anopheles mosquito infuses Plasmodium sporozoites into the bloodstream during a blood meal (11). The injected sporozoites home to the liver and infect hepatocytes within an hour of infection, where they multiply and differentiate into merozoites (12, 13). These merozoites are released into the bloodstream, where they further invade RBCs and multiply by schizogony over a period of 48 hours (in case of P. falciparum and P. vivax), to form the next generation of merozoites. P. vivax can form dormant hypnozoites in the liver, which may release merozoites and cause disease months or years later. P. falciparum, on the other hand, does not have the ability to form hypnozoites and differentiates completely from liver-stage schizonts to merozoites. It takes only a few seconds for merozoites to invade new blood cells, so merozoites spend most of their life inside RBCs and are relatively protected from the immune system. Within the RBCs the parasite differentiates and multiplies asexually, periodically leading to rupture of the parasitized RBCs (Pf-RBCs) and release of fresh merozoites in the blood circulation. It is during this blood stage, that infected person develop the periodic fevers, chills and other symptoms of malaria. Following RBC invasion, merozoite matures successively into ring (24 hr post invasion), trophozoite (24–36 hr) and schizont (36–48 hr) stages during which Pf-RBCs display decreased membrane deformability (14), become spherical, and develop cytoadherence
properties responsible for parasite sequestration in the post-capillary venules of different organs (15).

Fig. 2. Schematic representation of the malaria parasite’s life cycle

Some of the blood-stage parasites differentiate into male and female gametocytes, which are picked up by the mosquito during a blood meal. In response to signals in the mosquito midgut, the gametocytes release male and female gametes by a process referred to as exflagellation. The gametes undergo fertilization in the mosquito midgut and form a zygote. The zygote transforms into an ookinete, which traverses the midgut epithelium of the mosquito to form an oocyst on the outer wall of the midgut. The oocyst matures to release thousands of sporozoites that invade salivary glands and are ready to be injected into the vertebrate host again thus completing the parasite’s life cycle.
**Protein Trafficking in Eukaryotic cells**

The compartmentalization of eukaryotic cells has considerable functional advantages, but requires elaborate mechanisms to ensure that nascent proteins are correctly targeted to the appropriate compartment. Transport of proteins and lipids along the endocytic or secretory pathways is a hallmark of eukaryotic cells. Intracellular traffic to distinct destinations is very selective. The secretory system allows cells to regulate delivery of newly synthesized proteins, permitting membranous organelles to maintain distinct identities throughout the life of the cell (16).

**General features of the secretory pathway**

Eukaryotic cells use multiple intracellular secretory pathways to transport newly synthesized proteins from the endoplasmic reticulum (ER) to their final destinations. Nascent proteins destined for the secretory pathway often contain an N-terminal signal sequence (16), which directs them to the ER. At the ER exit sites, proteins destined to further compartments in the secretory pathway, leave the ER packaged into transport vesicles and are directed towards the Golgi, where they fuse to form and maintain the cis-Golgi compartment. Upon processing and maturation, they progress to the trans-Golgi compartment and thus form the trans-Golgi network (TGN). Proteins destined for secretion are sorted into one of two types of vesicles according to the secretion pattern of the protein. Some proteins are secreted continuously and these vesicles immediately move to the plasma membrane, fuse and release their contents by exocytosis. Other proteins are exported by regulated secretion. These proteins accumulate in the secretory vesicles, awaiting a stimulus for controlled exocytosis. This directional membrane flow is balanced by retrieval pathways, which bring membrane and selected proteins back to the compartment of origin (16).
General mechanisms of vesicular transport

The principal feature of vesicular traffic is that the vesicles bud from a donor compartment ("vesicle budding") by a process that allows selective incorporation of cargo into the forming vesicles while retaining resident proteins in the donor compartment ("protein sorting"). The vesicles are subsequently targeted to a specific "acceptor" compartment ("vesicle targeting"), into which they unload their cargo upon fusion of their limiting membranes ("vesicle fusion"). Cargo proteins that are loaded into the vesicles are thereby delivered to the acceptor compartment (17). The biogenesis of transport vesicles is initiated through the recruitment of large multi-subunit protein complexes termed coats. These coats are supra-molecular assemblies of proteins that are recruited from the cytosol to the nascent vesicles. The coat in general performs two principal functions: it integrates specific membrane proteins into the vesicle membrane that help to select the cargo protein and curves and deforms the membrane, eventually leading to the release of coated transport vesicles from the donor compartment (18). Vesicle budding and cargo selection at different stages of the exocytic and endocytic pathways is mediated by different coats and sorting signals. Dependent on the type of coats, the vesicles are recruited to a specific membrane compartment within the cell. The first coats to be identified and characterized contained a scaffold protein, clathrin, as their main constituent (19, 20). Clathrin coats were initially assumed to participate in most, if not all, vesicular transport steps within the cell. However, subsequent studies demonstrated that the function of these coats was restricted to post-Golgi locations including the plasma membrane, the TGN, and endosomes [Fig. 3]. Later, Rothman and Schekman, for the first time demonstrated the involvement of non-clathrin coats during vesicular transport in the early secretory pathway (21, 22). One of these coats, COPII, is now known to mediate export from the ER to either the ER-Golgi intermediate compartment (ERGIC) or the Golgi complex (22), while another coat, COPI, is involved in intra-Golgi transport and retrograde transport from the Golgi to the ER (23).
During the formation of a vesicle, a limited set of coat proteins carry out a programmed set of sequential interactions that lead to budding from the parent membrane, uncoating, fusion with a target membrane, and recycling of the coat components. The formation of vesicle is initiated by sequential binding of at least five soluble components, including the small GTPase Sar1p, and two cytosolic hetero-dimeric protein complexes, Sec23p/Sec24p (inner coat) and Sec13p/Sec31p (outer coat) [Fig. 4A] for COP II coated vesicles (24), whereas COPI coat is a heptameric protein complex [Fig. 4B], composed of α-COP (160 kDa), β+β'-COP (110 kDa), γ-COP (98 kDa), δ-COP (61 kDa), ζ-COP (20kDa), ξ-COP (36kDa) (25).
At the acceptor membrane, before the vesicle fusion the coat is discarded. The next steps in the fate of a transport vesicle involve proteins of the Rab GTPase family. Different Rabs
are localized on distinct vesicles and organelles. Rabs also interact with different proteins, loosely termed as Rab effectors that perform diverse function from vesicle budding to vesicle transport and vesicle docking at the target membrane (26). Due to their specific localization and their ability to regulate the Rab effectors, Rabs provide an element of regulation to the vesicle-trafficking machinery (26). The final step in vesicle trafficking is the fusion of a vesicle with its target membrane, which is mediated by a family of proteins termed SNARES (soluble NSF-attachment receptors) (17). SNAREs are integral membrane proteins present on both vesicle and target membranes, and can form very stable complexes, termed SNAREPIN complexes. The formation of a SNAREPIN complex pulls the vesicle and target membrane together [Fig. 5] and may provide the energy to provoke fusion of the lipid bilayers (17).

![Fig. 5. Schematic representation of vesicle budding and fusion (adapted from Bonifacino and Glick, 2004)](image-url)
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**Trafficking signals**

Signals for targeting to various compartments are encoded in the protein sequence called sorting signals, which function much like ‘Zip codes’. The process of cargo selection is driven by the direct interaction of coat proteins with specific sorting signals that are responsible for directing uptake of cargo molecules into a budding vesicle (27, 28).

Alternatively, in the absence of any sorting signal, secretory cargo can be selectively recruited to targeted vesicles by means of transmembrane receptors/adaptors that carry an ER export signal in their cytosolic domain recognized by the COPII coat (29). Various ER export motifs have been defined, including aromatic, hydrophobic and di-acidic motifs (30, 31, 32).

<table>
<thead>
<tr>
<th>Proteins</th>
<th>Functions</th>
<th>Signals</th>
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<tbody>
<tr>
<td>Sys1p</td>
<td>Golgi protein; high copy suppressor of ypt6 mutants</td>
<td>DLE</td>
</tr>
<tr>
<td>Gap1p</td>
<td>General amino acid permease</td>
<td>DLD</td>
</tr>
<tr>
<td>VSV-G</td>
<td>Envelope glycoprotein of vesicular stomatitis virus</td>
<td>YYDIE</td>
</tr>
<tr>
<td>KvlL.1</td>
<td>Inwardly rectifying potassium channel</td>
<td>FCYENE</td>
</tr>
<tr>
<td>Kvit.1</td>
<td>Inwardly rectifying potassium channel</td>
<td>VLEVDVETD</td>
</tr>
<tr>
<td>Pme28p</td>
<td>Pheromone-regulated membrane protein</td>
<td>FF</td>
</tr>
<tr>
<td>ERGIC53</td>
<td>Mammalian type I transmembrane lectin; ER export receptor for subset of glycoproteins; homologous to yeast Emp46p and Emp47p</td>
<td>FF</td>
</tr>
<tr>
<td>hmp248</td>
<td>p24 family member; putative ER export receptor; homologous to yeast Erv25p and Emp24p</td>
<td>FF</td>
</tr>
<tr>
<td>Erv46p</td>
<td>ER vesicle transmembrane protein; part of a complex with Env41p</td>
<td>FY</td>
</tr>
<tr>
<td>Erv41p</td>
<td>ER vesicle transmembrane protein; part of a complex with Env46p</td>
<td>IL</td>
</tr>
<tr>
<td>Emp46p</td>
<td>Type I transmembrane lectin; cycles between the ER and the Golgi; homologous to Emp47p</td>
<td>YYM, LL</td>
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<tr>
<td>Emp47p</td>
<td>Type I transmembrane lectin; cycles between the ER and the Golgi; homologous to Emp46p</td>
<td>LL</td>
</tr>
<tr>
<td>Env25p</td>
<td>p24 family member; putative ER export receptor; part of a complex with Emp24p</td>
<td>FF, LV</td>
</tr>
<tr>
<td>Emp24p</td>
<td>p24 family member; ER export receptor for Gas1p; part of a complex with Env25p</td>
<td>FF, LV</td>
</tr>
<tr>
<td>SedSp</td>
<td>Golgi t-SNARE</td>
<td>YNNSNPF, LMLME</td>
</tr>
<tr>
<td>Bet1p</td>
<td>ER-Golgi v-SNARE</td>
<td>LASLE</td>
</tr>
<tr>
<td>GalT2</td>
<td>Golgi enzyme</td>
<td>RR</td>
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<tr>
<td>GalNAcT</td>
<td>Golgi enzyme</td>
<td>RR</td>
</tr>
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Information was obtained from Barlowe (2003) and Giraudo and Mecocci (2003). Underlining indicates known key residues.

**Table 1. ER export signals**

**p24 proteins as cargo adaptors**

The p24 proteins are type 1 transmembrane proteins of ~25 kDa and were first identified as abundant constituents of the COPI and COPII vesicles that operate in the early secretory pathway. Because of their abundance, conservation throughout evolution, and the fact that they shuttle between the ER and Golgi compartments in transport vesicles (33, 34, 35), the p24 proteins are fundamental constituents of vesicles, perhaps acting as cargo receptors. The large luminal region containing the GOLD (Golgi dynamics) domain
includes two conserved cysteine residues and is the most likely candidate for mediating the cargo-receptor interaction (36, 37, 38), although other domains potentially aid the interaction (39). In the membrane proximal part of the GOLD domain, there is a region that includes a set of heptad repeats of hydrophobic residues indicative of a coiled-coil protein interaction domain. Additional characteristic features of these proteins include a C-terminal transmembrane domain and a short cytosolic tail, typically 12–15 residues, containing sequence motifs known to specify interactions with vesicle coat proteins (37). Members of the p24 family have been discovered in a wide range of eukaryotes, including mammals and other vertebrates (40). These proteins are commonly divided into four subgroups based upon phylogenetic analysis: α, β, γ and δ (41). The p24 family is composed of 8 members in yeast, 5 in Caenorhabditis elegans, and at least 7 in mammals. In mammals, the seven members that have been identified to date are gp25L (p24α1), GMP25 (p24α2), p24 (p24β1), p26 (p24γ1), Tp24 (p24γ2), gp27 (p24γ3) and p23 (p24δ1) (42). Increasing evidences suggest that p24 proteins from different families form a functional heteromeric complex, which had been shown to require the coiled-coil stretch (39, 43, 44) and these interactions may be necessary for their proper sorting and function (45). One of the bottlenecks in understanding the mechanisms responsible for proper localization/function of p24 family members is that in vivo studies of individual members are difficult. Ectopically over expressed p24 proteins appear to be retained in the ER and fail to reach their destination (33, 34, 35, 41). However, GFP-tagged p23 or p24 have been shown to be properly targeted, at least in some cells, when expressed at low levels (33). In addition, proper localization of some p24 family members could only be observed after co-transfection with cDNAs encoding for several proteins of the family (34, 41).

*Interactions with Coat proteins (COPI and COPII).*

Several lines of evidence indicate that p24 proteins bind cytoplasmic coat proteins (41). The first p24 protein to be identified, gp25L (hp24α1), was isolated as an abundant
calnexin-binding protein of the ER (46). This protein displays three discrete motifs in its cytoplasmic domain. The first is a diphenylalanine (FF) motif, which is conserved throughout the p24 family and in some members is a part of a larger motif, F/YXXXXF/Y. The FF motif has been shown to be required for transport out of the ER and mediates a direct interaction with coat components of COPII complex (41). The second motif, K(X)KXX, situated closer to the C-terminus, is also conserved among the p24 proteins but to a lesser extent. This motif is known to be involved in interaction with COPI coat subunits both in-vitro and in-vivo (36, 37, 39). All p24α proteins display a typical K(X)KXX motif, however members of other subfamilies show variations of this motif. For example, p23 (hp24γ1) and p24 (hp24β1) have one additional amino acid or two lysines substituted with arginines, respectively, rendering these less efficient in COPI binding (41). A third motif, situated at the extreme C terminus of p24α, β, and δ proteins, shows similarities with the φφ motif involved in endocytic sorting. The role of the φφ motif in p24 proteins is less clear, although it has been suggested that they enhance exit rates out of the ER (47).

Functional role of p24 proteins within the secretory pathway

As Cargo Adaptors: In yeast cells, the finding that p24 defects causes slowed kinetics of secretion of a subset of cargo proteins (36, 39, 45), led to the suggestion that p24 proteins might act as cargo receptors, the function of which is to ensure the concentration of cargo into COPII-coated vesicles. Deletion studies in yeast have revealed that disruption of Emp24p (p24β), caused delayed transport of a GPI-anchored protein, Gas1p (36). Recently, p24 have been directly implicated in mediating the transport of GPI anchored proteins in yeast and mammals (42, 48). Other evidences point to a role of p24s in COPI-mediated retrograde vesicle traffic from the Golgi to the ER. In yeast, p24 defects caused elevated secretion of the ER protein Kar2p, normally retrieved from the Golgi via retrograde trafficking of the Erd2 receptor (49). Members of p25 and p23 have also been implicated in selective retrieval of tyrosine phosphatase, TC48 to its ER localization, which otherwise
translocates to the Golgi complex along the secretory pathway (50). Although this suggests that the p24 proteins play crucial roles in the fidelity of vesicular transport between the ER and the Golgi, available data from mutational analysis have provided a somewhat murky picture regarding how p24 proteins work. Studies using a strain lacking all eight members of the family show that p24 proteins are not essential for vesicular transport in yeast (51), yet p23 are required for early embryonic development in mice (52).

**Vesicle biogenesis:** Additionally, Emp24p have also been proposed to play a structural role in vesicle assembly and budding (37) as well as in ER exit site formation during protein trafficking (53). Since the p24 proteins function as coat protein receptors as well as cargo receptors [Fig. 6], it has been proposed that they might function in vesicle biogenesis too (54). Consistent with this, it has been observed that p24 defects allow the bypass of the requirement for one component of the COPII coat, Sec13p, suggesting the existence of a regulatory mechanism linking cargo recruitment with the completion of COPII coat assembly. Previous studies also indicated that p23 and perhaps other p24 proteins (41), play a structural, morphogenic role in Golgi organization and/or biogenesis (35, 55).

![Fig. 6. Schematic representation of vesicle biogenesis dependent on sequential interaction of adaptor with cargo and coat proteins](image)

The p24 proteins may also serve as quality control agents that prevent misfolded or otherwise aberrant proteins from being loaded into vesicles. Dominant-negative mutations
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in the *C. elegans* p24 locus *sel-9* suppress mutations that produce mutant forms of members of the LIN-12/NOTCH family of cell fate determinants, allowing the aberrant proteins to be transported to the plasma membrane of the cell (56). Recently, it has been shown that the loss of p24 function in *Drosophila melanogaster* causes a stress response and increased levels of NF-κB-regulated gene products (57), further suggesting their role in quality control mechanisms.

Taken together, this abundant and well-conserved family of membrane proteins is likely to play an important role in trafficking, protein sorting and quality control between ER and Golgi apparatus.

**Protein trafficking in *P. falciparum***

*Beyond the parasite confines*

Malarial parasites not only have a complex life cycle alternating between a vertebrate and an invertebrate host, but also are capable of developing within highly specialized RBCs—a challenge met by only a few other intracellular pathogens. The evolutionary decision to inhabit terminally differentiated denucleated host cells places the parasite in a difficult situation. It severs itself from the external nutrient supply essential for growth and reproduction, and renders itself vulnerable to clearance by the spleen of the host organism, which removes senescent and infected erythrocytes from the circulation. To circumvent these problems, the parasite modifies the host cell by exporting its own proteins into the cytoplasm and plasma membrane of the erythrocytes, establishing immune evasion mechanisms and creating new permeation pathways for nutrient uptake. The vital modifications of the host cell including cytoadherence, stabilization of the host cell cytoskeleton and nutrient acquisition are attributed to a group of nearly 400 parasite proteins exported into the quiescent RBC (58-62).

Within the host cell, the parasite resides within a membrane-bound compartment, referred to as the parasitophorous vacuole (PV) (63). Prior to translocation to the
erythrocyte membrane, the *Plasmodium* proteins bound for export traverse two membranes, the plasma membrane of the parasite and parasitophorous vacuolar membrane (PVM). Secreted proteins released at the parasite plasma membrane, but destined for a location within the host cell thus find themselves "trapped" in the lumen of the parasitophorous vacuole, and must therefore be translocated across the PVM. The erythrocyte lacks a secretory apparatus and so cannot contribute in trafficking of proteins. To transfer proteins across the PVM and then through the erythrocyte cytoplasm, the parasite establishes a protein-trafficking system beyond the bounds of its own plasma membrane. Parasite derived membranous structures called as Maurer's cleft are thought to play a vital role in protein trafficking within the host cytosol (64).

Mechanistically, the trafficking of parasite-encoded proteins destined to the PV and beyond can be divided into two steps: secretion of the protein into the PV and the transport of the protein past the PVM. Several lines of evidence suggest that the first step occurs through the ER-based secretory pathway. The secretory pathways of *P. falciparum* have been suggested to be similar to that of higher eukaryotes, with proteins entering the ER, based on presence of a functional N-terminal ER targeting signal (58, 65, 66). In an experimental system investigating the export of histidine-rich protein II (HRPII) fusion to GFP, it was shown that the signal sequence of HRPII could direct the secretion of GFP to the PV (67). Conversely, an HRPII–myc fusion that lacked a signal sequence was not secreted, but detected in the parasite cytosol, indicating that a functional signal sequence is required for export (67). Apart from classical ER signal sequences, several exported proteins, such as glycophorin-binding protein-130, KAHRP, and ring expressed surface antigen (RESA), have also been shown to contain an unusual recessed signal sequence, located more than 50 amino acids from the N-terminus (68). Once into the ER, the proteins destined to PV or beyond are trafficked along the secretory pathway in vesicles that eventually fuse with the parasite plasma membrane, releasing their cargo into "extracellular space". Alternatively these vesicles have been proposed to deliver the proteins directly into
host cytosol at 'contact points' between the PPM and PVM [Fig. 7]. This mechanism of direct protein delivery has been referred to as "one step model" (69, 70). However, studies on the transport of the resident PVM protein PfExp1 revealed that the demonstrated topology of this protein upon co-translational insertion into the ER cannot be reconciled with the observed topology in the PVM (70, 71) and hence a "two step model" was proposed.

![Protein trafficking models in the infected erythrocyte](image)

**Fig. 7.** Protein trafficking models in the infected erythrocyte (Adapted from Charpian and Przyborski, 2008)

According to the 'two step model', the soluble parasite proteins destined for the host erythrocyte pass transiently through the lumen of the PV before being secreted into the RBC (72, 73). The second step of export, past the PVM, has been shown to be governed by a conserved pentameric transport sequence referred to as the Plasmodium Export Element (PEXEL) or Host Targeting signal (HTS) (59, 60). The HT signal is located at N-terminal within 100 amino acids following the signal sequence of exported proteins and is
highly conserved. The consensus sequence is R/KxLxQ/E and is found in both soluble and membrane proteins which are exported into the RBC cytosol (59, 60). The discovery of this element was a major advance as it allowed predictions of the exported proteome of P. falciparum and other Plasmodium species, and also suggested the presence of a central portal through which most or all exported proteins must pass. In support of this hypothesis, it has been shown that to translocate across PVM into the erythrocyte cytosol, the proteins pass through a membrane-bound protein-conducting channel (PCC) within the PVM, in an unfolded state. This PTEX complex (translocon of exported proteins) has been shown to translocate proteins across the PVM in PEXEL dependent manner (74).

Within the parasite confines

Apart from exporting proteins beyond its confines, at least a dozen distinct protein destinations are known to exist within the parasite precincts. Protein trafficking within the parasite is more complicated by the fact that the parasite harbors a number of unusual organelles such as the apicoplast, the digestive vacuole and the apical organelles (75-78).

 Trafficking to the food vacuole

The food vacuole of parasite contains a number of proteases involved in hemoglobin digestion (79). All the known food vacuole (FV) proteases bear a putative N–terminal signal peptide, signifying that they are transported through the ER and a prodomain that might have a role in targeting from the ER to the FV. Recently, the targeting of two FV-targeted proteases has been studied in detail and they have been shown to be involved in trafficking to the FV by two different routes: Plasmepsin II travels via ER to the food vacuole (76), whereas the dipeptide aminopeptidase1 does not traffic directly to the food vacuole but instead accumulates in the PV before moving to the FV (76, 79).
Protein trafficking to the apicoplast

The apicoplast is surrounded by four membranes and most apicoplast proteins are nuclear-encoded and require a bipartite N-terminal extension made up of a signal peptide and a transit peptide (80). Earlier work has shown that the signal peptide resembles a classical eukaryotic signal peptide and mediates targeting into the ER, whereas the transit peptide mediates further steps of trafficking to the apicoplast lumen. Interestingly, it has been shown that apicoplast-targeted proteins do not traverse the Golgi but rather divert straight from the ER to the apicoplast (81, 82).

 Trafficking to apical organelles

*Plasmodium*, like other apicomplexan parasites, possesses three sets of morphologically and functionally distinct secretory organelles—rhoptries, micronemes and dense granules (DGs). Consistent with microscopic observations that apical organelles are synthesized from Golgi-derived vesicles, most proteins destined for rhoptries, micronemes or DGs carry a classical secretory signal sequence (83). Those that lack an N-terminal signal are presumed to be translocated into the ER by courtesy of an internal hydrophobic domain (78, 84). Immunofluorescence microscopy on trophozoite stage parasites has demonstrated that proteins *en route* to apical organelles transit through compartments of the secretory system (84, 85). Trafficking of these proteins can be blocked by Brefeldin A and incubation at low temperature, further confirming the involvement of the ER and Golgi trafficking machinery (85, 86).

Protein sorting

At some point after their entry into the ER, proteins targeted to intracellular destinations must be sorted from each other and from other proteins in the secretory pathway such as those being trafficked to the parasite surface or host cytosol. In general, many of the sorting steps take place along the early secretory pathway [Fig. 8]. The presence of a
"classical" secretory pathway within the parasite is indicated by the identification of homologues of a number of trafficking components such as those associated with ER: PfERC and PfBip (87-89); the COPI protein, PfδCOP (90); and the retrieval receptor, PfERD2 (91). Analysis of the genome has also revealed homologues of components of the signal recognition particle, the translocon, the signal peptidase complex and many proteins involved in vesicle budding, docking, and fusion (4). Further several components of the COPII coat have also been identified: PfSar1, PfSec23, PfSec31, and PfSec13 (92-95).

On the other hand, the Golgi appears to be quite rudimentary in the parasite with a single discoid cisterna close to the nucleus. Recent advances in the morphological analysis however, have suggested the spatial separation of cis-Golgi from the trans-Golgi compartment (95-97).

Fig. 8. Putative trafficking pathways in a P. falciparum iRBC (Adapted from Cooke et al., 2004)
Although several components of classical vesicle mediated pathway like PfSar1, PfNSF, PfSec23 and PfSec31 were initially localized both within the parasite as well as to Maurer's clefts in the host cell cytosol [Fig. 8], recent studies using GFP fusions of PfSar1 suggested an exclusive localization of this component to the parasite ER (98). Similarly, analyses of sub-cellular distribution of GFP-tagged SNARE orthologs showed association of PfSyn5p and PfSec22p with ER and Golgi compartments, and were also localized to other punctuated structures within the parasite plasma membrane. This study argued against any involvement of SNAREs in vesicular trafficking within the host cell compartment (99).

In addition, PfSec13p, PfSec12 and PfSec24a were also observed to be restricted to defined areas of the ER membrane juxtaposed to the Golgi apparatus (95, 100). Such evidences point to a very restricted role of vesicular trafficking machinery beyond the parasite confines. On the other hand, this also emphasizes to explore the role of classical vesicle mediated pathway in the initial protein sorting and trafficking, which is crucial for accurate targeting of proteins to their appropriate destinations.

Understanding the protein sorting machinery deployed by the parasite to resolve intrinsic traffic jams and faithfully target the proteins to their correct destinations is challenging and should lead to a better understanding of yet undefined aspects of parasite cell biology. For in depth understanding of the trafficking machinery and to study the involvement of classical vesicle mediated pathway in protein sorting, we explored the role of p24 adaptors in the parasite. To this end, the localization and trafficking of p24 adaptors within the secretory pathway was studied, and their role in organizing the ER export was investigated. Further, the possible cargo proteins carried by these adaptors were also delineated.