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Hardly Vacuous: the Parasitophorous Vacuolar Membrane of Malaria Parasites

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Abstract

When a malaria parasite invades a host erythrocyte, it pushes itself in and invaginates a portion of the host membrane, thereby sealing itself inside and establishing itself in the resulting vacuole. The parasitophorous vacuolar membrane (PVM) that surrounds the parasite is modified by the parasite using its secretory organelles. To survive within this enveloping membrane, the organism must take in nutrients, secrete wastes, export proteins into the host cell and eventually egress. Here, we review current understanding of the unique solutions *Plasmodium* has evolved to these challenges and discuss the remaining questions.

Keywords

Plasmodium; parasitophorous vacuole

Parasitophorous Vacuolar Membrane of Malaria Parasites

While it is not yet one hundred years after the flurry of activity by the earliest electron microscopists that defined the concepts and paradigms of cellular membrane topology, their hypotheses have moved well beyond theories – they are generally established now as the law that governs protein and vesicle trafficking within cells. And given how different the cytoplasm and extracellular fluid are, it stands to reason that the separate surfaces that make up the membrane bilayer should also be very different; indeed, the plasma membrane has asymmetrical composition of inner and outer leaflets. Measured by area, the membrane that surrounds the related parasite *Toxoplasma* tachyzoite as it pushes itself into a host cell prior to pinching off is nearly completely derived from host cell membrane[1]. Likewise, the lipids of the membrane enveloping the malaria parasite are derived, at least partially, from the erythrocyte membrane[2]. Thus it is appealing to imagine that the malaria parasite,

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Plasmodium, also simply invaginates a portion of the host membrane, sealing itself inside and establishing a parasitophorous vacuole (PV) that retains the inverted asymmetry of all endosomal membranes (Figure 1). But however appealing, it cannot be this simple. For one thing, the **parasitophorous vacuolar membrane (PVM, see Glossary)** that surrounds the malaria parasite is unique in its paucity of host protein -- most integral membrane proteins may be actively excluded and lipids may be added during its formation[3,4]. For another, PVM topology seems backwards: whereas most host cellular vacuoles would be bound by a membrane transporting proteins (synthesized in the host cellular cytoplasm) *into* the vacuolar lumen, the PVM transports proteins (synthesized in the parasite cytoplasm) *out* of the vacuole and *into* the host cell cytoplasm, a feature also seen in many intracellular bacterial vacuoles[5,6]. But unlike bacterial vacuoles in nucleated cells, malaria parasites do not have to face destruction by the host cell and instead focus on obtaining nutrients and evading extracellular immunity. Structure and function have been adapted by *Plasmodium* to fit its particular needs.

Biogenesis and Structure

During invasion, the parasite modifies the PVM by secreting components of the rhoptry organelles[7,8]; once inside, discharge of dense granules contributes to formation of the PVM as well[9–11]. Undoubtedly, lipid alterations occur with invasion [7,12,13] but how and when these alterations occur, whether the lipid changes are symmetric or asymmetric, and how lipid composition changes throughout the life cycle of the parasite are unknown. In fact, there is not even consensus on the lipid composition of the PVM. Clearly, the mechanisms of lipid homeostasis in this organism are poorly understood.

PVM/PPM Relationship

The PVM is closely apposed to the **parasite plasma membrane (PPM)** and recent evidence suggests attachment points between the two[14]. Specifically, soluble proteins in the parasitophorous vacuole are excluded from portions of the vacuolar space. Also, knockdown of the single-pass membrane protein EXP1 causes increased separation of PVM from the PPM[15]. The abundant single-pass early transcribed membrane proteins (ETRAMPS) could participate in formation of attachment points between the two membranes as well[16]. Further, blockade of protein export across the PVM causes blebbing of sections of PVM into the erythrocyte cytoplasm, away from the PPM but tethered at foci where the two membranes remain together[17,18]. A similar phenotype has been observed in liver-stage parasites[19].

Tubovesicular Network

In contrast to the blebbing described above, normal extensions of PVM into the erythrocytic space do occur, called the **tubovesicular network (TVN)**[20]. This network is speculated to provide membrane for the formation of **Maurer's clefts**[21,22], a Golgi-like structure out in the host erythrocyte that is responsible for trafficking exported parasite effector proteins to the erythrocyte surface and perhaps vesicular destinations as well. The extra surface area provided by the TVN may also allow the remarkable gross shape fluctuations in early-stage parasites that facilitate growth and ameboid activity within the erythrocyte[23,24].

Domains

Careful observation of the PVM reveals a heterogeneous lateral distribution of PVM components, alternatively described as beaded, patchy, or piebald[14,23,25], with limited protein flow between foci as assessed by photobleaching experiments[26]. The integral membrane protein **EXP2** by live fluorescence microscopy is proximal to a soluble parasitophorous vacuole marker (signal peptide-tagged mRuby3), while the PPM lipid transporter PfNCR1 (Niemann-Pick type C-related protein 1)[27] anticorrelates and may correspond to attachment domains with lipid flow between PVM and PPM[14].

Protein Targeting

While it has been established that a signal sequence is sufficient to get a reporter into the PV lumen[26], PVM targeting may be less straightforward. A study on the gametocyte protein Pfs16 suggested that its N-terminal signal sequence plus a C-terminal motif containing a hydrophobic stretch was enough to target a GFP reporter to the PVM, and possesses a motif also found in other PVM proteins such as Exp1 and Exp2 [28]. The protein PfAK2 was shown to contain an N-terminal sequence sufficient for targeting a GFP reporter to the PVM, likely anchored by myristate and palmitate moieties, and this was presented as an alternate targeting sequence [29]. Still more work remains to be done to understand PVM targeting.

Function

Any consideration of PVM function must start with an inevitable comparison to a related apicomplexan parasite *Babesia*, whose PVM disappears soon after formation[30,31]. The PVM is not an obligate structure of every intracellular apicomplexan organism; fundamentally intracellular parasites can thrive without a continuous macromolecular barrier to host cytosol[31,32]. But unless the malaria parasite PVM is vestigial, i.e. a remnant of the invasion process, its continuing presence in all malaria species indicates a selective advantage in the cytoplasm of erythrocytes, and its existence mandates that proteins evolve to modify the PVM in order to carry out essential functions for the replication of viable daughter cells: the PVM must facilitate intake of nutrients, excretion of wastes and export of protein effectors for manipulation of the environment outside the parasite (Figure 2, Key Figure). Ultimately, the PVM creates a physical barrier for the daughter cells to overcome when it is time to leave. The parasite needs to destroy the PVM in order to **egress**.

Nutrient Acquisition and Waste Excretion

The clearest problem with retaining a continuous spheroidal bilayer as a retaining wall is that it is a barrier to the flux of nutrients and waste materials. From the bloodstream the parasite requires pantothenate for CoA synthesis, glucose for glycolytic metabolism, isoleucine for protein synthesis (most amino acids are obtained from hemoglobin but there is no isoleucine in human hemoglobin), a purine and certain lipids[33–35]. Pathways of lipid acquisition in the infected RBC are poorly defined but for most hydrophilic small molecules, entry into the infected erythrocyte is via parasite-derived new permeation pathways[36], especially **PSAC**[37,38], a channel whose components are exported by the parasite out to the erythrocyte membrane[39–41]. Glucose, in contrast, appears to predominantly enter via the host glucose transporter Glut 1[42]. These molecules must then pass through the PVM to

get to specific transporters at the PPM. At the PVM, there is a channel established by the parasite protein EXP2[17] that allows nutrients smaller than 1.4 kDa to pass through[43,44]. Molecular assignment of the nutrient channel activity to EXP2 was based on observation of altered electrical gating properties in EXP2 mutants lacking a C-terminal charged region[17]. Knockdown of this channel protein is lethal to the parasite[17]. A similar channel exists in *T. gondii*[45]. In addition to facilitating nutrient import, this channel is thought to allow export of wastes, especially the glycolytic product lactate, as well as excess amino acids, ATP and glutathione (ATP and glutathione may be exported to maintain host cell homeostasis and oxidative balance)[46–48].

Hemoglobin Degradation

Hemoglobin consumption is also required for parasite maturation, as alluded to above, both to acquire small molecules (such as amino acids and heme) and to maintain osmotic homeostasis[47,49–51]. Hemoglobin is brought in by means of an endocytic apparatus called the **cytostome** that spans the PVM and PPM membranes[52], effecting delivery to the acidic **digestive vacuole** where proteolysis, heme sequestration and amino acid/peptide transport to the parasite cytoplasm take place. Formation of the cytostome is poorly understood, but could take place at the membrane domains where the PVM and PPM are closely apposed and likely contiguous[14].

Effector Protein Export

The constraint of a PVM necessitates that the parasite translocate protein effectors across this membrane into the erythrocyte. Exported proteins contribute to the aforementioned PSAC nutrient channel at the infected erythrocyte surface. Others form knob structures under the erythrocyte surface to cluster variant surface antigens called PfEMPs that it sends out to mediate adherence to the vascular endothelium (thereby avoiding the spleen). Still others form microvesicles, modify erythrocyte cytoskeleton rigidity or manipulate vascular tone[53,54]. The translocon required for export (**PTEX**) was first identified as a PVM complex with a putative pore protein (EXP2) and a AAA⁺-ATPase that could potentially unfold translocon cargo (HSP101)[10,55]. HSP101 was recently shown to have unfoldase activity[56]. The core complex also contains an adaptor protein, PTEX150. Knockdown experiments have demonstrated the essential roles of all three components in effector export[57,58]. Establishment of the PSAC nutrient channel is impaired by translocon disruption[58]. Accessory translocon components (PTEX88 and TRX2) are non-essential in cultured parasites but could play roles in export of particular effectors[59,60]. The translocon-associated protein RON3 has recently been shown to be essential for glucose acquisition and protein export[61], and further work is required to determine the mechanism of RON3 action and the nature of its interaction with PTEX. How the translocon determines which proteins get exported and which remain in the PV or its membrane remains unknown. There is information important for export at the N-terminus of mature effector proteins, but no clear motif or recognition mechanism has been identified.

The cryoEM structure of the malaria parasite translocon[62] shows a twisted hexamer of HSP101 docked into the funnel-shaped EXP2 heptameric channel, attached via a heptameric PTEX150 adapter (Figure 3). Two conformations of the translocon were seen, with a

compressed or extended HSP101 multimer interacting with offset, unraveled single chains of cargo protein inside the translocon, suggesting a processive threading or compaction mechanism of translocation. It is interesting that Plasmodium EXP2 appears to have a dual role in nutrient uptake and in protein export[14]. These appear to be different functions, perhaps involving different multimeric complexes[17]. The *T. gondii* EXP2 ortholog Gra17 has only the nutrient channel function[45]. Both parasites possess HSP101s, but it seems that Plasmodium has evolved the adaptor protein PTEX150 to recruit EXP2 and HSP101 to allow protein translocation. Recent work shows that the protein translocation function of the translocon can be competent when nutrient import channel activity is insufficient[63].

Egress from the Host Cell

A final challenge for the PVM-enveloped malaria parasite is that at the end of its growth and replication, it must get out of the PVM (and host erythrocyte) to invade new red blood cells. To do so, about 10 minutes before egress[64], the parasite initiates the cGMP/Ca⁺⁺-triggered secretion of **subtilisin-like protease 1 (SUB1)** into the PV[65–67] (Figure 4). There, SUB1 activates egress effectors called SERAs (a family of cysteine proteases) and MSPs (a family of merozoite surface proteins involved in invasion as well as egress) [67–70]. SUB1 knockout prevents PV swelling, rupture and dissolution[67]. MSPs and SERAs are thought to play later roles in exit from the host cell, so how SUB1 mediates PVM destruction is unclear. Knock down or chemical inhibition of the aspartic protease **plasmepsin X** prevents maturation of SUB1 and phenocopies SUB1 knockout, placing this enzyme at the top of the proteolytic cascade (Fig 4)[71,72]. Plasmepsin X inhibition is a promising avenue for antimalarial chemotherapy.

Unanswered Questions

PVM Replenishment

Although we are starting to learn about this important membrane surrounding the intraerythrocytic malaria parasite, much remains unknown. The Unanswered Questions section begins with a paradox – while PVM depletion is expected after invasion due to the need for significant membrane area to form Maurer’s clefts and to transit hemoglobin to the digestive vacuole, in fact the PVM grows during the first day of intraerythrocytic development. Thus, we do not understand how this membrane is replenished; there must be mechanisms of membrane recycling and rebuilding. Various lipid components are synthesized, scavenged or a combination of the two[73,74]. Details of lipid acquisition for a number of lipid species is still sketchy. Lipid gradients between membranes are maintained[75], so there must be homeostatic mechanisms that are largely undiscovered. PfNCR1 is a lipid transporter that appears to contribute to membrane lipid maintenance[76] from its topologically bewildering position in the PPM. Its substrate is most likely cholesterol but this hypothesis has not been formally demonstrated. The return journey for a lipid molecule from the digestive vacuole back to the PVM, while seemingly essential, remains to be elucidated.

PVM Formation

The contributions of merozoite organelle secretion (rhoptries, dense granules) in formation of the PVM has been studied extensively in *Toxoplasma*[77], but the surface has barely been scratched in *Plasmodium*, in part due to the smaller size of the host cell, the parasite, and its granules.

PVM Structure

We do not understand the structure of the domains of the PVM or how the attachment points to the PPM are formed. The abundant ETRAMP proteins and the related protein EXP1 are likely to be structurally important[78], but more work needs to be done.

Hemoglobin Ingestion

The ingestion of hemoglobin through a cytostome that spans the PVM and PPM is understood only at a rudimentary morphological level[79]. Molecular components and mechanisms remain to be discovered.

Specificity of Protein Export

As alluded to previously, the puzzle of the specificity of protein export across the PVM is yet to be solved[80], as well as the roles for most translocated protein effectors. More generally, the presumed plasma membrane exocytosis of translocon substrates by fusion of Golgi-derived cargo vesicles is not understood, nor are the mechanisms by which newly synthesized transmembrane proteins, which should be retained by the PPM, get to a translocon that is in the wrong membrane – the PVM.

Concluding Remarks

Given that *Babesia* escapes from its PVM, as discussed above, the mystery remains as to why intraerythrocytic malaria parasites go to the trouble of maintaining a PVM, given the attendant challenges of access to the host cell. In the related apicomplexan parasite *Toxoplasma gondii*[81,82] and in liver-stage *Plasmodium*[83], the PVM protects the parasite from the host cell, which is trying to kill it via GTPase-mediated attack, apoptosis and/or autophagy. Protein components of the PVM in these cases are mostly different from those of intraerythrocytic *Plasmodium*[78]. The parasite does devote a significant portion of its genome to establishing the Maurer's clefts[84] that allow protein sorting in the host cell to get virulence determinants to the surface. Perhaps the PVM is needed mainly to extend into the host cell and provide membrane for Maurer's cleft formation, though the PVM is maintained even after this organelle is formed. In certain respects, the maintenance of the PVM creates a compartment, an organelle out of the parasite that looks like a cytoplasmic inclusion to the host cell, and the parasite decorates its new house to its advantage. Alternatively, it could be that the parasite is protecting itself from something in the erythrocyte such as a protein, an RNA, an osmotic force. There is much that remains to be elucidated about PVM structure and function (see Outstanding Questions). We look forward to learning more about this fascinating outer covering of the intraerythrocytic malaria parasite that is itself an inner liner to the host erythrocyte.

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GLOSSARY

Cytostome

a mouth-like structure that spans the PVM and PPM and effects ingestion of hemoglobin by the parasite

Digestive Vacuole

the lysosome-like compartment where hemoglobin is degraded and heme is sequestered as hemozoin

Egress

the process of parasite escape from the host cell after replication and packaging of daughter cells (merozoites)

EXP2

a PVM protein that forms the channel component in PTEX for protein export and also forms a channel for nutrient import

Maurer's clefts

a membranous structure elaborated by the parasite in the cytoplasm of the host erythrocyte, involved in targeting exported effectors to locations in the host cell including the erythrocyte surface

Parasite plasma membrane (PPM)

the membrane that bounds the parasite

Parasitophorous vacuolar membrane (PVM)

the membrane that envelops the parasite as it enters the host erythrocyte

Plasmepsin X

an aspartic protease that processes and activates SUB1; its blockade prevents egress and positions it as an attractive drug target

PSAC

a parasite-genetically encoded channel at the host cell surface that facilitates nutrient uptake

PTEX

the translocon complex at the PVM that facilitates effector protein export

Subtilisin-like protease 1 (SUB1)

a proteolytic enzyme central to the process of egress

Tubovesicular network (TVN)

the membrane network that extends from the PVM and is thought to provide membrane for the Maurer's clefts

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Highlights

- Recent work suggests that the PVM is not a homogeneous membrane, but rather has domains that are specialized for transport and for lipid exchange between membranes
- The PVM is tethered to the PPM at distinct attachment points
- A translocon complex called PTEX exports protein effectors into the host erythrocyte
- The structure of this complex has been solved by cryoEM and appears to function by a threading/compaction mechanism
- The translocon pore protein, EXP2, has a separate function as a nutrient channel across the PVM
- Egress from the host cell requires a proteolytic cascade centered on SUB1, which is required for breaking down the PVM as well as rupture of the RBC

OUTSTANDING QUESTIONS

- How is the PVM replenished upon depletion from formation of Maurer's clefts and cytotomes?
- How does the malaria parasite alter the nascent PVM?
- How do PVM domains and attachment foci form?
- How does hemoglobin ingestion work?
- How is protein export specified? What do all these exported effectors do?
- Why does the parasite maintain a PVM?

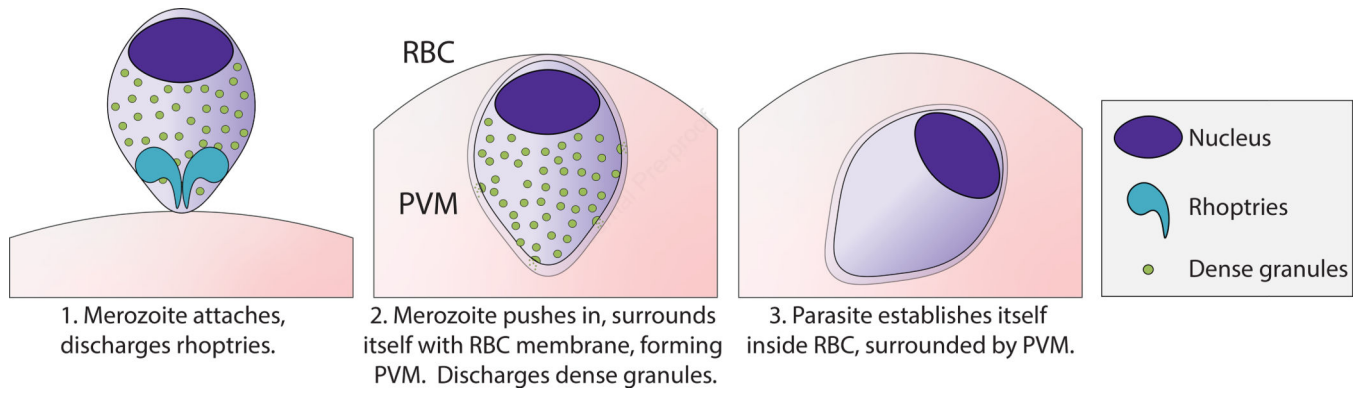


Figure 1. Formation of the PVM.

An infectious merozoite attaches to an erythrocyte, discharging rhoptries to facilitate invasion and add content to the recipient membrane. The merozoite pushes its way in, surrounding itself with RBC membrane and forming the PVM. It discharges dense granules to contribute to the PVM. The parasite then established itself inside the erythrocyte, surrounded by the PVM.

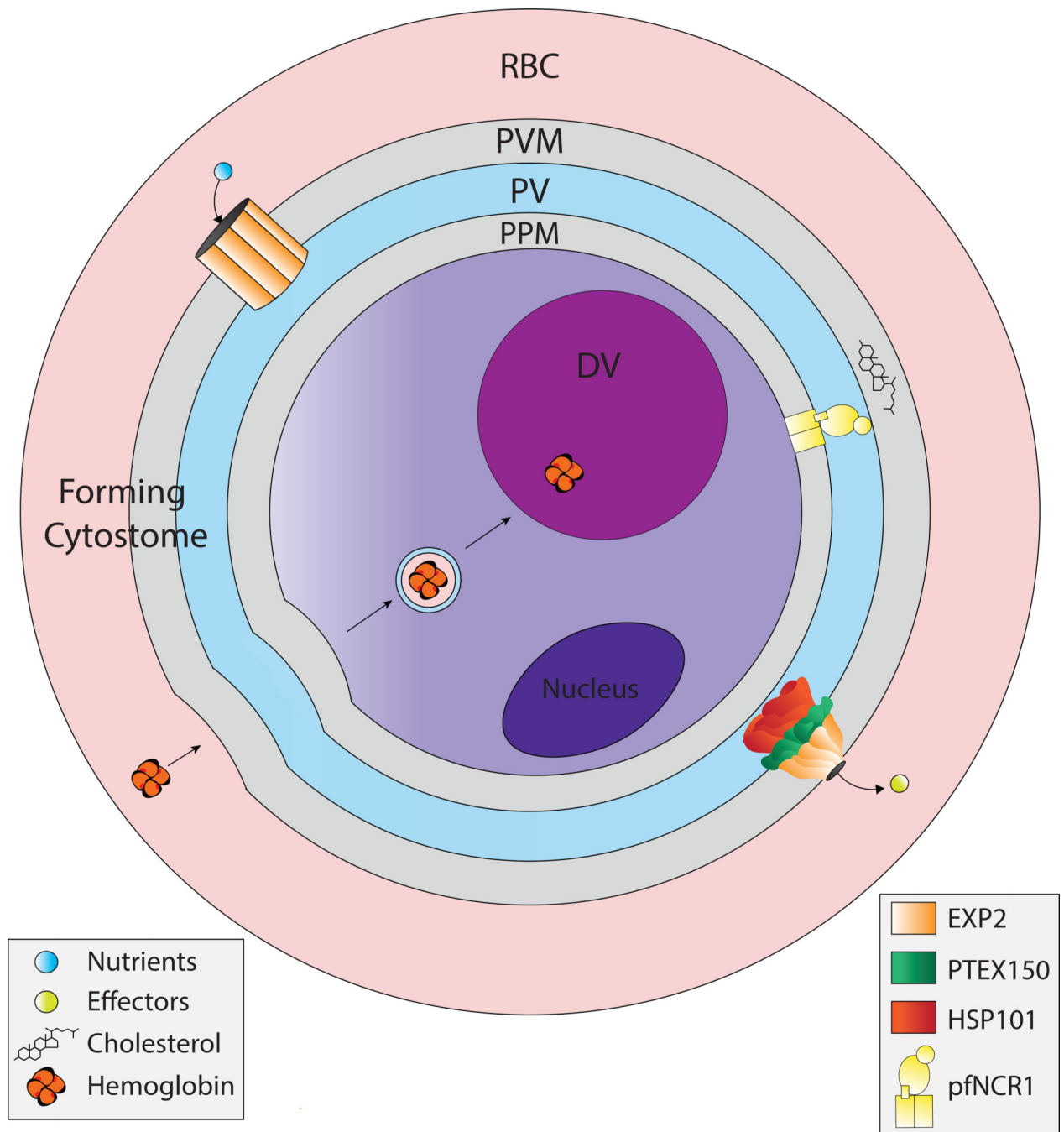


Figure 2, Key Figure. Functions of the PVM.

The PVM has a translocon complex called PTEX to export effector proteins into the host cell. There is a nutrient channel formed by EXP2 to import nutrients. It likely excretes wastes as well. EXP2 is depicted in the two separate macromolecular complexes, detailed in Figure 3. There is a forming cytostome that spans the PVM and PPM to ingest hemoglobin. There is a lipid transporter, PfNCR1, that contributes to membrane lipid homeostasis, likely by keeping cholesterol out of the plasma membrane. DV, digestive vacuole.

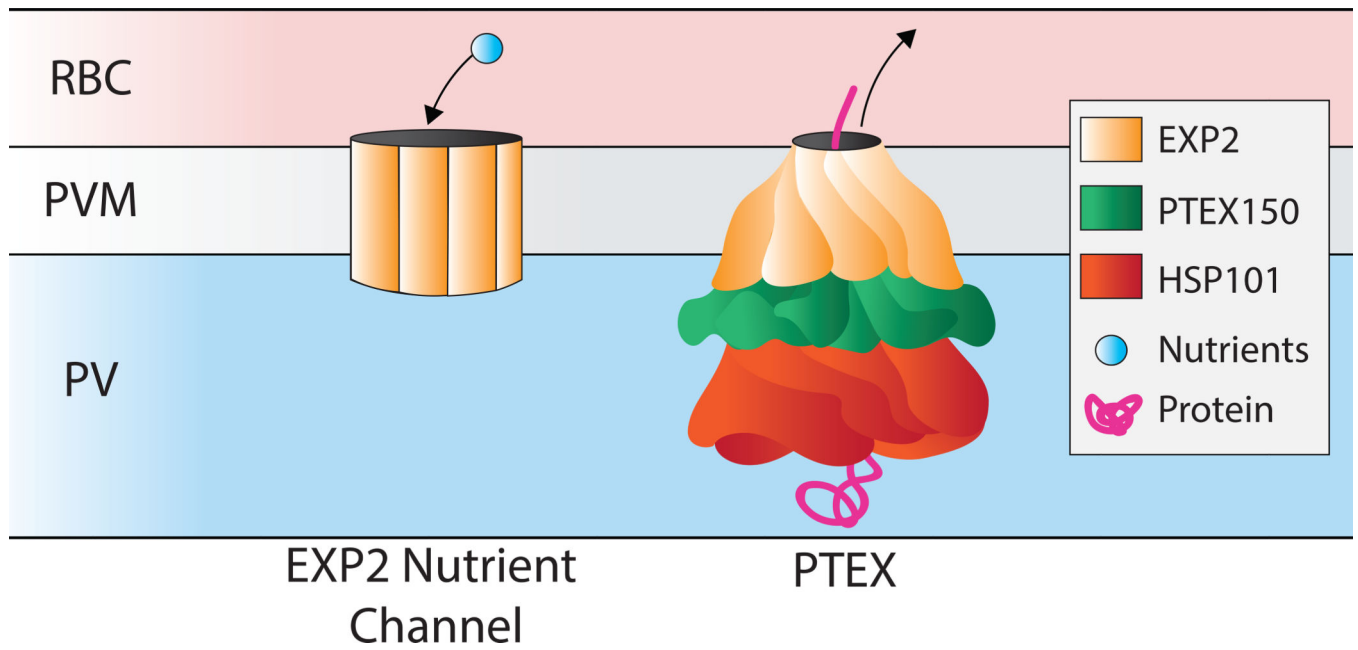


Figure 3. Dual functions of EXP2.

EXP2 forms a nutrient channel, and separately forms the PTEX translocation channel in association with Hsp101 and PTEX 150. EXP2 is depicted as a homomultimer for the nutrient channel, though tertiary and quaternary structural details are unknown and there may be other components of this complex that are undefined. For PTEX, EXP2 is shown as a twisted, funnel-shaped multimeric channel in association with other core components PTEX150 and HSP101, based on the cryo-EM structure[62].

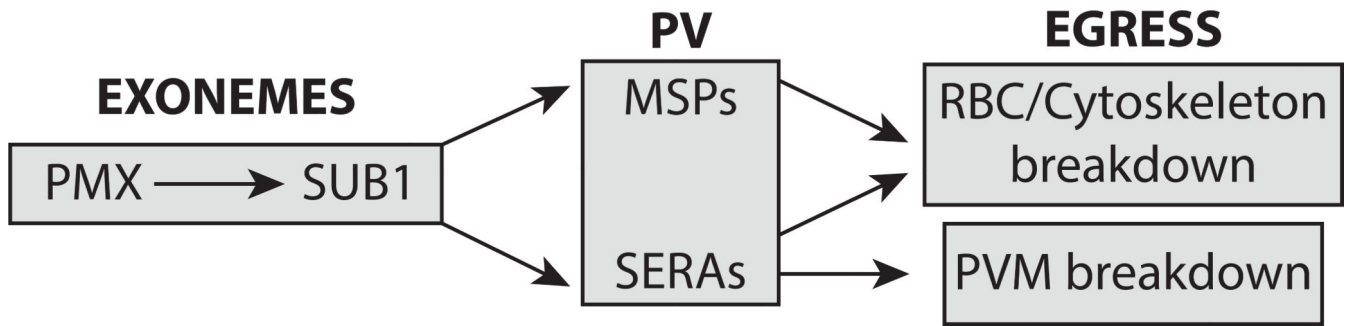


Figure 4. Proteolytic egress cascade.

Plasmepsin X (PMX) is the maturase for subtilisin-like protease 1 (SUB1), which activates cysteine proteases called SERAs and merozoite surface proteins (MSPs), leading to PVM, RBC membrane and cytoskeleton breakdown. The specific roles of these later effectors in the egress process are largely unknown.