Moreno Silvia (Orcid ID: 0000-0002-2041-6295)

Corresponding author mail id : smoreno@uga.edu



# The *Toxoplasma* Plant-Like Vacuolar Compartment (PLVAC)

Andrew J. Stasic[1], Silvia N.J. Moreno[2,3]*, Vern B. Carruthers[4], Zhicheng Dou[5]

[1]Department of Technology Development, Quantigen Biosciences, Fishers, IN 46038, USA.
[2]Department of Cellular Biology, University of Georgia, Athens, GA 30602, USA.
[3]Center for Tropical and Emerging Global Diseases and Department of Cellular Biology, University of Georgia, GA 30602-7400, USA.
[4]Department of Microbiology & Immunology, University of Michigan Medical School, Ann Arbor, MI 48109-5620, USA.
[5]Department of Biological Sciences, Clemson University, Clemson, SC 29634, USA.

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## Abstract

*Toxoplasma gondii* belongs to the phylum Apicomplexa and is an important cause of congenital disease and infection in immunocompromised patients. *T. gondii* shares several characteristics with plants including a non-photosynthetic plastid termed apicoplast and a multi-vesicular organelle that was named the plant-like vacuole (PLV) or vacuolar compartment (VAC). The name plant-like vacuole was selected based on its resemblance in composition and function to plant vacuoles. The name VAC represents its general vacuolar characteristics. We will refer to the organelle as PLVAC in this review. New findings in recent years have revealed that the PLVAC represents the lysosomal compartment of *T. gondii* which has adapted peculiarities to fulfill specific *Toxoplasma* needs. In this review, we discuss the composition and functions of the PLVAC highlighting its roles in ion storage and homeostasis, endocytosis, exocytosis, and autophagy.

*TOXOPLASMA gondii* infects approximately one-third of the world's population (Hill & Dubey 2002; Pappas *et al.* 2009) and is an opportunistic pathogen of immunocompromised patients like HIV-infected individuals, fetuses, and organ transplant

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recipients (Luft & Remington 1992; Weiss & Dubey 2009). The resulting pathogenesis of *T. gondii* infection is due primarily to tissue destruction originating from the fast-growing tachyzoite form, which engages in a lytic cycle that consists of multiple rounds of host cell invasion, replication, and egress (Black *et al.* 2000; Blader *et al.* 2015). *T. gondii* secretes proteins from specific apical organelles, micronemes and rhoptries, which mediate host cell attachment and invasion (Carruthers & Sibley 1997). Invasion is facilitated by mechanical extrusion of the conoid, a tubulin-based apical structure (Hu *et al.* 2002; Dos Santos Pacheco *et al.* 2020). After parasite replication, active egress of *T. gondii* ultimately results in lysis of host cells and can cause significant tissue damage (Frenal *et al.* 2017). Once outside host cells *T. gondii* tachyzoites are challenged by sharp changes in the concentrations of the surrounding ions and nutrients in addition to their exposure to the host immune response.

*Toxoplasma* possesses a lysosome-like organelle that was originally termed the plant-like vacuole (PLV) (Miranda *et al.* 2010) or vacuolar compartment (VAC) (Parussini *et al.* 2010), which we will rename PLVAC in this article. The PLVAC expresses several pumps and transporters that enable the storage of toxic ions like zinc, calcium, protons, and others. Also, the PLVAC expresses hydrolytic enzymes and functions as a digestive organelle similar to mammalian lysosomes and plant lytic vacuoles (Boller & Kende 1979). Like a plant lytic vacuole, the PLVAC contains the vacuolar-$H^+$-ATPase, the vacuolar-$H^+$-pyrophosphatase (V-$H^+$-PPase) and the tonoplast intrinsic proteins (TIP)-like aquaporins (Martinoia *et al.* 2007). In addition, the PLVAC is most noticeable as a single entity in extracellular and early intracellular stages of the tachyzoite and fragmented into multiple smaller components in intracellular stages. For these reasons it was proposed that the PLVAC helps parasites survive the sharp change in ionic composition of the surrounding milieu upon egress (Miranda *et al.* 2010).

**Molecular components of the PLVAC (Table 1)**

Several transporters and pumps that localize to the PLVAC have been characterized. The first description of this vacuolar compartment was based on the localization of the cysteine protease Cathepsin L, the vacuolar $H^+$-pyrophosphatase, and the water channel Aquaporin 1 (Table 1). The presence of several plant-like activities like the vacuolar proton pyrophosphatase (TgVP1), a vacuolar proton ATPase (TgV-ATPase), a cathepsin L-like protease (TgCPL), and an aquaporin (TgAQP1) explain the naming of plant-like vacuole or PLV. The organelle was also termed the vacuolar compartment or VAC reflecting its general vacuole-like characteristics (Fig. 1, inset).

**The *T. gondii* Cathepsin L or TgCPL,** is a cysteine protease and represents one of five papain-like cathepsins present in *T. gondii* (Dou & Carruthers 2011). TgCPL is first synthesized as an immature precursor, which is processed into a mature form that contains the key catalytic residues important for proteolytic activity (Huang et al. 2009;



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Larson *et al.* 2009). TgCPL defined the PLVAC compartment, a unique structure distinct from other apical organelles, but clearly associated with the exocytic and the endosomal system. TgCPL displayed a specific localization to a large vacuolar compartment usually found toward the apical end of the tachyzoite. The localization of TgCPL to the PLVAC in extracellular and newly invaded *T. gondii* tachyzoites was determined using specific antibodies (Parussini *et al.* 2010). Some co-localization of TgCPL was seen with the micronemal protein TgAMA1 (Parussini *et al.* 2010), indicating a possible link to the microneme pathway. Potential substrates for TgCPL were determined to be proTgM2AP, proTgMIC3 and proTgMIC6 (Parussini *et al.* 2010). Deletion of the TgCPL gene in RH *T. gondii* parasites caused an invasion defect, which was also seen with cysteine protease inhibitors (Teo *et al.* 2007; Larson *et al.* 2009; Chaparro *et al.* 2018). RHΔ*cpl* tachyzoites also showed diminished replication, which is recapitulated with protease inhibitors targeting TgCPL (Dou *et al.* 2013; Chaparro *et al.* 2018).

Although the dominant protease of the PLVAC is cathepsin L, other proteases like **cathepsin B, C and D** have been shown to co-localize with PLVAC markers. **TgCPB** maturation was dependent on TgCPL, unlike the self-maturation of many cathepsins (Dou *et al.* 2013). A deletion mutant of TgCPB did not show an evident growth defect and showed a limited role in the digestion of host proteins (McDonald *et al*. 2020). Concerning **TgASP1** (a cathepsin D-like aspartyl protease), the data support that its maturation also involves the activity of TgCPL, although other proteases are also likely involved. TgASP1 was not required for tachyzoite replication or digestion of host protein in the PLVAC (McDonald *et al.* 2020). We discuss further the role of PLVAC proteolytic activities in the chronic infection section.

**The vacuolar-$H^+$- pyrophosphatase (V-$H^+$-PPase)** is a membrane-associated proton pump that couples the hydrolysis of pyrophosphate (PPi) to the active transport of protons against the electrochemical gradient (Maeshima 2000). They are termed vacuolar because of their preferential localization to vacuolar compartments. These enzymes, which are absent in mammalian cells, have been mostly studied in plants in which their main role is the transport of protons into the plant vacuole (Drozdowicz & Rea 2001). V-$H^+$-PPases are also present in protists, including apicomplexan parasites like *T. gondii* and *Plasmodium falciparum* (McIntosh & Vaidya 2002). In *T. gondii,* the enzyme (TgVP1) was first localized in intracellular parasites to an endosome-associated compartment (named VP1 compartment), which accumulated the microneme protein MIC2 when the propeptide of its partner protein MIC2-associated protein (M2AP) was deleted (Brydges *et al.* 2006). This compartment is likely what is now termed the endosomal-like compartment or ELC (Jackson *et al.* 2013), which is probably a mixture of early and late endosomes (Parussini *et al.* 2010). However, VP1 can redistribute to the PLVAC in extracellular parasites (Miranda *et al.* 2010), potentially because of fusion of the ELCs with the PLVAC. This notable redistribution highlights the dynamic nature of the *T. gondii* endolysosomal system. The proton pumping activity of TgVP1 was inhibited by PPi

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analogues like imidodiphosphate (IDP) and aminomethylene diphosphonate (AMDP) and was stimulated by $K^+$ ions (Miranda *et al.* 2010). Both AMDP and other PPi analogues inhibited *T. gondii* growth (Rodrigues *et al*. 2000; Drozdowicz *et al*. 2003). Proton pumping activity was enriched in a subcellular fraction obtained after a subcellular fractionation followed by an iodixanol discontinuous gradient. Enrichment for this PLVAC marker was determined following the AMDP sensitive PPi hydrolysis activity (Miranda *et al.* 2010). The fraction was also enriched for other PLVAC markers like TgCPL and TgV-ATPase. In plants, the V-$H^+$-PPase and the V-$H^+$-ATPase are located in the same membrane (Rea *et al*. 1992) and, consistent with this, Miranda *et al.* (Miranda *et al.* 2010) observed an additive acidification activity stimulated by PPi and ATP in fractions enriched for the PLVAC.

Deletion of the TgVP1 gene in the *Δvp1* mutant caused defects in attachment and invasion of host cells, which correlated with defective microneme secretion and TgCPL maturation (Liu *et al*. 2014). In addition, the *Δvp1* mutant could not regulate osmotic stress and was less virulent to mice. The mutant was viable and able to grow *in vitro*, and the mild phenotype was attributed to a compensatory role of the TgV-ATPase.

**The vacuolar- $H^+$-ATPase (V-ATPase)**, a protein complex consisting of 14 different subunits, couples the hydrolysis of ATP to the pumping of protons across membranes, often into the lumen of acidic vesicles (Forgac 2007; Saroussi & Nelson 2009). The function and localization of the *T. gondii* TgV-ATPase were inferred through the characterization of the *a1* subunit (vha1), which is a 100-kDa integral membrane that spans both domains of the complex ($V_o$ and $V_1$) (Forgac 2007). The N-terminal domain connects $V_1$ and $V_o$ and stabilizes the complex during rotary catalysis. The C-terminal domain is membrane-embedded and is involved in proton transport (Wang *et al*. 2008). It had been demonstrated that the V-ATPase pumps protons to the extracellular milieu, which likely contributes to the homeostasis of intracellular pH and to the PM membrane potential (Moreno *et al.* 1998). Vha1 localized to both the plasma membrane and the PLVAC (Stasic *et al*. 2019). Conditional control of the expression of the *vha1* gene permitted the characterization of this essential gene. Conditional repression of vha1 caused defects in all the major steps of the lytic cycle, including maturation and secretion of micronemes, invasion, motility, and egress. Defects in invasion and $H^+$ pumping activity were evident earlier than the microneme secretion defect. Altered $H^+$ pumping impacted $Ca^{2+}$ regulation and signaling, which negatively impacted invasion (Stasic *et al.* 2019; Stasic *et al*. 2021). Depletion of vha1 negatively impacted the maturation and correct localization of several microneme proteins, most likely due to defective acidification of the compartment where their respective maturases are processed. A model was proposed based on the presence of two acidic endosome compartments expressing different levels of TgVP1. Acidification of both organelles would be important for either the maturation process of maturases and/or for their specific proteolytic activity on their substrates,

including essential adhesins secreted for invasion. The presence of more than one of these compartments could represent how lytic activity is regulated by limiting and/or allowing contact with substrates.

The PLVAC also expresses an aquaporin water channel, **TgAQP1,** which has high similarity to the plant aquaporins known as tonoplast intrinsic proteins (TIPs), found in lytic plant vacuoles (Martinoia *et al.* 2007). A motif analysis of representative proteins from the major intrinsic protein (MIP) family of aquaporins supports the close relationship between TgAQP1 and plant $\gamma$-TIPs (Miranda *et al.* 2010). Overexpression of the TgAQP1 gene permitted its visualization in the PLVAC using specific anti-AQP antibodies. The IFA signal of the AQP co-localized with TgCPL signal in extracellular parasites. The normal level of expression of the *TgAQP1* gene is most likely very low making it difficult to visualize endogenous levels of the protein. Deletion of the *TgAQP1* gene did not affect growth of *T. gondii*. No functional analysis was done of the TgAQP1 protein, but *T. gondii* overexpressing the TgAQP1 gene were more resistant to $HgCl_2$, an AQP1 inhibitor (Miranda *et al.* 2010).

**A zinc transporter (TgZnT)** was localized to vesicles (acidocalcisomes) that fuse with the PLVAC and mutant parasites for the TgZnT showed a retarded growth phenotype, which was exacerbated by increasing zinc concentrations in the extracellular milieu (Chasen *et al.* 2019), supporting a role for the transporter in zinc tolerance in extracellular tachyzoites.
Zinc is an essential element as it acts as a cofactor for a large number of enzymes (Coleman 1992). However, its cytosolic concentrations need to be tightly regulated as high sustained concentrations of zinc could be deleterious for all cells (Choi & Koh 1998; Kim *et al*. 2000; Sheline *et al*. 2000). Although the concentration of zinc in cells ranges from 0.1-0.5 mM, most of the metal is bound to proteins or sequestered into lysosomal compartments, and the cytosolic resting concentration of zinc is reported to be at picomolar levels (Outten & O'Halloran 2001; Woodier *et al*. 2015). It was proposed that the TgZnT in the PLVAC plays an essential role in the homeostasis of zinc and protection against its toxicity. The function of the transporter was validated by yeast complementation (Chasen *et al.* 2019).

**The chloroquine resistance transporter (CRT)** was localized to the digestive vacuole (DV) of *Plasmodium* parasites (Fidock *et al*. 2000), a lysosomal organelle and the site of hemoglobin degradation and chloroquine action (Fidock *et al.* 2000). Mutations in PfCRT resulted in reduced chloroquine accumulation in the DV causing parasites to become resistant to the drug (Martin *et al*. 2009). Although originally identified as the main basis for chloroquine resistance, later work determined that mutations in the PfCRT were associated with the parasite sensitivity to many antimalarials (Martin 2020). PfCRT belongs to a family of transporters termed the CRT family, which are found in all apicomplexan parasites, other protists, and plants. The *Arabidopsis* chloroquine-like



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transporter was localized to the plastid where it mediates glutathione transport (Maughan *et al.* 2010).

More recent studies characterizing *T. gondii Δcrt* mutants, showed a severely swollen PLVAC (Kannan *et al*. 2019; Thornton *et al*. 2019). The deletion of *TgCRT* arrested the separation of PLVAC from its precursor, the ELC, during parasite replication (Thornton et al. 2019). Also noted was aberrant localization of markers and down-regulated expression of several proteases that reside in the PLVAC (Thornton *et al.* 2019). These defects resulted in altered microneme secretion, invasion, and virulence. Additionally, TgCRT is speculated to serve as a transporter for small nutrient molecules as its native function, and accordingly the loss of TgCRT is expected to cause accumulation of solutes in the PLVAC and increased osmotic pressure, which further leads to the enlargement of the PLVAC. The studies supported the central role of TgCRT in controlling the PLVAC size and integrity through the movement of small molecules (osmolytes) from the organelle into the cytosol to maintain the PLVAC osmolarity.

A study characterizing the **phosphate transporter TgPiT**, determined its localization to the PLVAC, mainly to internal vesicles formed by the inward budding of its limiting membrane. TgPiT also labeled other cytoplasmic vesicles and the plasma membrane (Asady *et al*. 2020). Gene deletion and complementation determined that TgPiT is important for parasite survival, virulence, and osmoregulation. The *Δpit* mutant showed reduced cell volume and showed a reduction in the overall levels of polyphosphate (polyP) and phosphate (Pi) content despite an increase in the number of acidocalcisomes, the main site for polyP storage (Rodrigues *et al.* 2002). The *Δpit* mutant showed reduced acidic $Ca^{2+}$ release, which was attributed to reduced levels of polyPs. It is possible that the intraluminal membranes labeled with TgPiT are acidocalcisomes since acidocalcisomes have been shown to interact with the PLVAC (Miranda *et al.* 2010).

A report on the molecular characterization of the vacuolar type **$Na^+$/$H^+$ exchanger, TgNHE3**, showed its co-localization with TgVP1 in intracellular parasites, likely associated with the ELC (Francia *et al.* 2011). Extracellular tachyzoites of the TgNHE3 knockout mutant showed sensitivity to hyperosmotic shock, high sodium, and contained higher intracellular $Ca^{2+}$ concentration $[Ca^{2+}]_i$, which resulted in a reduced host invasion efficiency, presumably due to the observed reduced secretion of micronemes. The results suggested that the function of ELCs and PLVAC in extracellular parasites is analogous to those of the vacuolar compartments of plants and yeasts, providing the parasite with a mechanism to resist ionic fluctuations and, potentially, regulate protein trafficking.

**A plasma membrane type $Ca^{2+}$-ATPase (TgA1)** was characterized and determined to be of the vacuolar type, and it was localized with a homologous antibody to a large vacuolar compartment in extracellular *T. gondii* (Luo *et al*. 2001). This work was published prior to the characterization of the PLVAC, and it was suggested to be a large acidocalcisome. Considering that the PLVAC most likely stores $Ca^{2+}$, it is possible that

TgA1 is the $Ca^{2+}$ pump involved. This has not been clearly shown. A TgA1 knockout mutant showed a mild growth phenotype but had microneme and invasion defects and reduced virulence (Luo *et al*. 2005). These biological defects were attributed to reduced levels of polyphosphates and increased resting calcium levels.

Polyphosphate (polyP) is a ubiquitous polymer of tens to hundreds of phosphate residues linked by high-energy phosphoanhydride bonds and can reach millimolar levels in protozoan parasites (assuming distribution across the entire cellular volume and expressed as Pi monomer), while the concentration in host cells is at the micromolar level. PolyP plays essential roles in cells as a phosphate reservoir, energy source, chelator of metal ions, and possesses regulatory roles in cell metabolism and stress response, differentiation, and gene expression. PolyP was shown to be enriched in acidocalcisome fractions. Considering that these organelles fuse/interact with the PLVAC as shown in a number of images (Fig. 2B-c from (Miranda *et al.* 2010)), most likely the PLVAC is a store of polyP as it has been shown for the yeast vacuole (Li & Kane 2009).

Studies in yeast showed that the synthesis of polyP, is catalyzed by a five subunits protein complex also known as the Vacuolar Transporter Chaperones or VTC1-5 (Cohen *et al.* 1999; Ogawa *et al*. 2000; Desfougeres *et al*. 2016). Two genes with homology to the yeast VTC proteins ScVtc2p (**VTC2**) and ScVtc4p (**VTC4**) were identified in *T. gondii* and we have shown that the VTC4 subunit co-localizes with the V-$H^{+}$-ATPase to acidocalcisomes and the PLVAC (Stasic *et al.* 2021). As polyP is negatively charged, it is most likely involved in chelating positively charged ions like $Ca^{2+}$, $Zn^{2+}$ and others. Alkalinization of acidic compartments containing polyP would activate their hydrolysis causing the release of $Ca^{2+}$ or other ions (Rodrigues *et al.* 2002), which implicates polyP in the homeostasis of positively charged ions.

## The PLVAC and its relationship to other vacuoles

The PLVAC in *Toxoplasma*, the digestive vacuole (DV) in *Plasmodium*, and the yeast vacuole in *Saccharomyces cerevisiae* share many features of the lysosome, such as storage of acidic hydrolytic enzymes and an acidic environment that facilitates degradation of proteins. Vacuoles in fungi (Klionsky *et al.* 1990) and plants (Marty 1999) share common features with mammalian lysosomes as they are highly dynamic and constantly changing and re-modeling with the cell cycle stages and growth conditions (Weisman 2006; Bandyopadhyay *et al*. 2014; Zhang *et al.* 2014; de Marcos Lousa & Denecke 2016). Mammalian lysosomes are smaller and more numerous (Novikoff *et al*. 1956; de Duve 2005; de Marcos Lousa & Denecke 2016) while most fungal vacuoles are larger and present in smaller numbers (Weisman 2006). Plant vacuoles, however, are large compartments and they can fill up to 90% of the plant cell volume (Owens & Poole 1979). One important function that is shared by lysosomes and other vacuoles is their lytic degradation activities performed by several specific hydrolases. For this function,



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these compartments need to maintain an acidic pH, between 4.5-5.5 for lysosomes and 5.5-6.2 for yeast and plant vacuoles (Preston *et al*. 1989; Martinez-Munoz & Kane 2008; Li & Kane 2009; Mindell 2012; Martiniere *et al*. 2013). The pH of the DV was estimated at ~5.2 (Kuhn *et al*. 2007), but the pH of the PLVAC has not been quantitatively determined. The presence of both proton pumps, TgVP1 and the V-$H^+$-ATPase in the PLVAC compartment, represents a similar arrangement to the plant vacuole. Neither yeast nor the mammalian lysosome expresses the V-$H^+$-PPase (Rea & Poole 1993) which is found in plants, bacteria, and some protists and it is absent in the Opisthokonta supergroup of eukaryotes (Rea & Poole 1993). There is no evidence for the expression of the mammalian lysosomal marker LAMP in any apicomplexan parasite (ToxoDB).

The PLVAC in *T. gondii* was shown to be involved in the digestion of host cytosolic proteins during the acute infection (Dou *et al*. 2014), mirroring hemoglobin degradation in the malarial DV in blood-stage infections (Francis *et al*. 1997). The PLVAC also functions in the turnover of parasite organelles via autophagy during chronic infection (Di Cristina *et al*. 2017). TgCPL was shown to be the major hydrolase for degradation functions (Larson *et al.* 2009; Parussini *et al.* 2010; Dou & Carruthers 2011; Dou *et al.* 2014) and, as noted above, it also proteolytically activates other PLVAC-localized proteases like TgCPB and TgASP1 (Dou *et al.* 2013; McDonald *et al.* 2020). In the *Plasmodium* DV a cascade of hydrolases carries on polypeptide degradation. Three falcipains (cathepsin L-like cysteine proteases) and four plasmepsins (cathepsin D-like aspartic proteases), which are endopeptidases, initialize the cleavage of hemoglobin into polypeptide fragments (Rosenthal 2004; Bonilla *et al*. 2007), followed by subsequent cleavages via falcilysin ($Zn^{2+}$-dependent metalloprotease) (Murata & Goldberg 2003), dipeptidyl aminopeptidase I (PfDPAP1, cysteine protease), aminopeptidase P ($Mn^{2+}$-dependent metalloprotease), and M1-family aminopeptidase ($Zn^{2+}$-dependent metalloprotease) (Dalal & Klemba 2007; Ragheb *et al.* 2011; Rosenthal 2011). In addition, plasmepsins are proteolytically activated by falcipains in the malarial DV. Unpublished data from the Dou lab showed that a *T. gondii* cathepsin C-like protease (TgCPC1), equivalent to PfDPAP1, was localized to the PLVAC. In the yeast vacuole, seven proteases were characterized, including three metalloproteases, three serine proteases, and one aspartyl protease (Hecht *et al*. 2014; Parzych & Klionsky 2019). Two endopeptidases, PEP4 (aspartyl endoprotease) and PRB1 (serine endopeptidase), act as major proteases that undergo self-cleavage and mature the other vacuolar proteases (Klionsky *et al.* 1990; Van Den Hazel *et al.* 1996; Hecht *et al.* 2014).

It is presently not clear how the hydrolysis products generated in the PLVAC and DV are transported out into the cytosol. Both organelles express a CRT (Fidock *et al.* 2000; Warring *et al*. 2014); however, the natural function of the CRT protein in apicomplexan parasites is still not entirely defined. A few *in vitro* studies reported that recombinant PfCRT transports a variety of substrates, such as 4 to 11-residue peptides derived from hemoglobin or other erythrocyte proteins, L-arginine, L-lysine, L-histidine, glutathione,

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and iron ions (Patzewitz *et al*. 2013; Juge *et al*. 2015; Bakouh *et al*. 2017; Shafik *et al*. 2020). PfCRT is an essential gene (Zhang *et al*. 2018), indicating its important role in malaria parasites. This contrasts with the function of the TgCRT in *T. gondii* as mutant parasites are viable (Kannan *et al*. 2019; Thornton *et al.* 2019).

These lysosome-like organelles are acidified by the V-$H^+$-ATPase complex (Yamashiro *et al*. 1990; Hayashi *et al.* 2000; Saliba *et al.* 2003; Stasic *et al.* 2019) and in addition in *S. cerevisiae* an NHE family of $Na^+/H^+$ exchanger (Nhx1), which also transports $K^+$, was shown to regulate both luminal and cytoplasmic pH and control vesicle trafficking out of the endosome (Brett *et al*. 2005). In addition, the PLVAC and DV express a V-$H^+$-PPase, for proton pumping into the vacuole (Luo *et al*. 1999; Saliba *et al.* 2003; Liu *et al.* 2014). The import of negatively charged phosphate to the PLVAC by the $Na^+$-phosphate co-transporter may help neutralize accumulated positive charges derived from proton translocation (Asady *et al.* 2020).

All three vacuoles, yeast, DV and PLVAC, serve as $Ca^{2+}$ stores in their respective cells (Biagini *et al*. 2003; Li & Kane 2009; Miranda *et al.* 2010). TgA1 in *T. gondii* would be responsible for the uptake of $Ca^{2+}$ into the PLVAC (Luo *et al.* 2001), while the yeast vacuole expresses two transmembrane proteins, PMC1 ($Ca^{2+}$-ATPase ortholog) and VCX1 ($Ca^{2+}/H^+$ exchanger) for $Ca^{2+}$ uptake with PMC1 having higher affinity. A $Ca^{2+}/H^+$ exchanger was described in *T. gondii* (Guttery *et al*. 2013), but its localization was not definitively determined.

## Biogenesis and dynamics

The PLVAC is a dynamic structure and its morphology changes along the parasite's lytic cycle. In extracellular and newly invaded stages, the organelle exists as a single large vacuole that becomes fragmented during intracellular replication, based on TgCPL staining (Miranda *et al.* 2010; Parussini *et al.* 2010). Intracellular fragmentation of the PLVAC was dependent on the cell cycle (Parussini *et al.* 2010). Parasites that remained extracellular for prolonged times (> 1 h) retain a coalesced PLVAC (Miranda *et al.* 2010). The signaling molecules or signaling pathways required for the formation and fragmentation of PLV are not yet known.

In *Toxoplasma*, the organelles that form earlier parts of the endolysosomal system are not well defined. As mentioned earlier, a mixture of endolysosomal organelles and vesicles was named the endosomal-like compartment (ELC) (Jackson *et al.* 2013), which likely includes early and late endosome, and a series of intermediary vesicles (Fig. 1). The ELC was proposed as a precursor in the biogenesis of the PLVAC, as well as of other secretory organelles like microneme, rhoptries, and dense granules (Jimenez-Ruiz *et al*. 2016; Sangare *et al*. 2016; Venugopal & Marion 2018). The classical endocytic trafficking pathway utilizes the class c core vacuole/endosome tethering factor (CORVET) complex and the homotypic fusion and vacuole protein sorting (HOPS) complex that regulates

early to late endosome and late endosome to lysosome trafficking, respectively (Mizuno-Yamasaki *et al.* 2012; Numrich & Ungermann 2014). The CORVET and HOPS complexes are associated with early endosome and late endosome/lysosome/vacuole markers, respectively. BLAST searches of the *T. gondii* genome reveal the presence of orthologs for 4 core components of the CORVET and HOPS complexes, including Vps11, Vps16, Vps18, and Vps33 (Morlon-Guyot *et al*. 2015). Genes for the small GTPase proteins associated with early endosome and late endosome, Rab5 and Rab7, and the corresponding guanine nucleotide exchange factor (GEF), Vps9 for Rab5 and Mon1/Ccz1 for Rab7, are also encoded in the *T. gondii* genome. Based on these observations, it is predicted that *T. gondii* uses Rab5, Vps9, and the CORVET complex to coordinate the trafficking from the *trans*-Golgi network to early endosomes, and switch gears to Rab7, Mon1/Ccz1, and HOPS complex to achieve early-to-late endosome/PLVAC transition. Interestingly, *T. gondii* encodes three Rab5 orthologs (Rab5A, 5B, 5C), and one Rab7. Both Rab5A and Rab5C are well localized to ELCs (Kremer *et al*. 2013; Sakura *et al*. 2016), whereas the plant-like Rab5B is only partially co-localized with Rab5A and Rab5C and to the parasite cell membrane (Kremer *et al.* 2013). TgRab7 is observed in both ELCs and PLVAC (Miranda *et al.* 2010; Parussini *et al.* 2010). As a core component of both CORVET and HOPS complexes, Vps11 is associated with the PLVAC and ELC (Morlon-Guyot *et al.* 2015). Conditional knockdown of Vps11 caused defective biogenesis of multiple organelles in the parasites, including PLVAC, rhoptries, micronemes, and dense granules (Morlon-Guyot *et al.* 2015). Two proteins that interact with the CORVET and HOPS complexes in the parasites were identified (Morlon-Guyot *et al*. 2018). One shows low similarity to the known Vps8 subunit, an effector in the CORVET complex, while the other one carries a BEACH (beige and Chediak Higashi) domain, named a BEACH domain-containing protein (TgBDCP). Vps8 and TgBDCP co-localized with Rab5, but not Rab7, suggesting that they are probably within the CORVET complex. Surprisingly, TgBDCP was co-localized with TgCPL, indicating that it may be directly involved in the biogenesis of the PLVAC or the traffic of some proteins to the PLVAC. The conditional knockout of both TgVps8 and TgBDCP impaired the formation of the PLVAC (Morlon-Guyot *et al.* 2018).

Repression of the V-$H^+$-ATPase lead to a fragmented PLVAC in extracellular tachyzoites (Stasic *et al.* 2019). Interestingly, the genetic deletion of TgCRT causes enlarged PLVAC, and the swollen organelle contained markers of both PLVAC and ELC (Thornton *et al.* 2019), indicating that the biogenesis of the PLVAC was arrested in the Δ*crt* mutant. Due to a significant volume increase in the Δ*crt* PLVAC, it was speculated that the acidity within the enlarged PLVAC would be reduced. Both findings suggested that the pH inside the PLVAC is critical for the vesicular trafficking within the endolysosomal system in *T. gondii.*

**Functions of the PLVAC**



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## Degradative functions of the PLVAC

The *T. gondii* PLVAC possesses many characteristics found in typical lysosomes including being populated by cathepsin proteases, which are commonly involved in degrading cellular proteins or proteins originating from endocytic events. *T. gondii* expresses six cathepsins including five papain-like cysteine proteases (TgCPL, TgCPB, and TgCPC1-3 (Dou & Carruthers 2011)) and one cathepsin D-like protease (TgASP1) (Shea *et al*. 2007; McDonald *et al.* 2020). TgCPL and TgCPB, have been localized to the PLVAC (**Fig.1**) (Miranda *et al.* 2010; Dou *et al.* 2013). TgCPC1 and TgCPC2 were reported to be secreted in the parasitophorous vacuoles (PV) and TgCPC3 is reportedly only expressed in the sporozoite stage (Que *et al*. 2007). In eukaryotes, cathepsin proteases are translated as preprocathepsins where the prepeptide is removed in the ER and the procathepsin is trafficked to the early endosome or lysosome. In the acidic environment of the lysosome, the procathepsin undergoes proteolytic processing. In *T. gondii*, TgCPL and TgCPB are synthesized as inactive procathepsins, trafficked to the PLVAC, where they are matured at low pH (Parussini *et al.* 2010; Dou *et al.* 2013). It was reported that a Δ*cpl* knockout strain accumulated host expressed GFP in the PLVAC (Dou *et al.* 2014). Recently, it was determined that host-derived proteins are endocytosed in as little as 7 min post-invasion, trafficked to the PLVAC, and digested in under 30 min (McGovern *et al*. 2018). Ablation of TgCPL in the Prugniaud strain, which is competent for differentiation to bradyzoites, showed that the PLVAC was a major player in proteolytic activity in the chronic stage (Di Cristina *et al.* 2017). Additionally, the PLVAC was shown to play a role in autophagy as undigested organelles appeared in the PLVAC in the Δ*cpl* mutant (Di Cristina *et al.* 2017). Collectively, these results showed that the PLVAC serves as a digestive organelle and that TgCPL, and possibly TgCPB, are active proteases able to digest some host-derived proteins.

## Protein trafficking and maturation through a hybrid endo/exocytic system

In mammalian and yeast cells, endocytosis begins by delivering cargo to the early endosome (marked by Rab5), followed by traffic to the late endosome (marked by Rab7), and finally fusion with the mature lysosome (also marked by Rab7) (Galvez *et al*. 2012). In plants, however, endocytosed cargo can transit through the trans-Golgi network (TGN) first before being routed through the early endosome and finally ending in the lysosome (Viotti *et al*. 2010). More recent work in yeast has also identified the TGN as an early and recycling endosome (Day *et al*. 2018). However, beyond the TGN the endocytic systems of mammalian or model systems typically do not intersect extensively with their exocytic systems, with a few exceptions in specialized cell types.

A difficulty in understanding the endocytic pathway in *T. gondii* was the inability for chemical dyes to be endocytosed and a lack of known receptors or trackable ligands usually involved in the endocytic process. There are some reported instances of endocytosed cargo shown to be internalized by *T. gondii*, but the mechanism(s) remains

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unknown (Nichols *et al*. 1994; Botero-Kleiven *et al*. 2001). More recent work showed that intracellular *T. gondii* is also able to endocytose host-derived proteins in an intravacuolar network-dependent manner, but the mechanism(s) and site of entry into the parasite remain unclear (Dou *et al.* 2014). These reports collectively suggested that *T. gondii* is capable of endocytic events, but much still needs to be studied about this process. *T. gondii* possesses a *trans*-Golgi network (TGN), ELCs, and the PLVAC and proteins important for endocytic trafficking like clathrin, dynamin, Rab5 and Rab7 are expressed (Tomavo *et al*. 2013). In addition, *T. gondii* expresses the plant-like TgVP1 and TgAQP1 which indicates that endocytic trafficking may resemble that of plants (Pieperhoff *et al*. 2013).

The PLVAC is a multi-vesicular highly dynamic compartment and its formation and fragmentation suggested constant fusion and budding of internal vesicles. Rab7, usually associated with late endosomes, co-localized with the PLVAC marker TgVP1 in intracellular parasites, supporting the role of the PLVAC in the endocytic pathway (Bucci *et al*. 2000). Recent reports suggested that the ELCs and the PLVAC are major compartments of the endocytic system (Dou & Carruthers 2011; McGovern *et al.* 2018). In addition, ingested host proteins made their way through the ELCs (possibly via the TGN) and were finally trafficked to and digested in the PLVAC (McGovern *et al.* 2018).

Exocytic trafficking destined to the rhoptries and micronemes, proceeds through the TGN and ELCs, and involves clathrin, dynamin and Rab5 (Harper *et al*. 2006; Sloves *et al*. 2012; Kremer *et al.* 2013; Pieperhoff *et al.* 2013; Sangare *et al.* 2016; Venugopal & Marion 2018). Ablation of the dynamin-related protein DrpB, the sortilin-like receptor (TgSORTLR), or the clathrin heavy chain, TgCHC1, resulted in loss of micronemes and rhoptries organelles (Breinich *et al*. 2009; Sloves *et al.* 2012; Pieperhoff *et al.* 2013). TgCHC1 was localized to the Golgi where it most likely plays a role in the stabilization of the Golgi as its ablation resulted in abnormal Golgi (Pieperhoff *et al.* 2013). HOPS and CORVET complexes, which are essential for the early to late endosome transition, lysosome biogenesis, and endolysosomal trafficking (Solinger & Spang 2013) are also critical for the biogenesis of all secretory organelles (rhoptries, micronemes, and dense granules) (Morlon-Guyot *et al.* 2018). Collectively, these findings suggest that the *T. gondii* endocytic system is also a conduit for proteins destined for exocytosis.

Additional evidence supports a role for the ELCs and PLVAC in the proteolytic maturation of microneme proteins (McGovern *et al.* 2018; Stasic *et al.* 2019). Disruption of the PLVAC and ELC proton pump TgVP1 resulted in defects in microneme secretion, microneme organelle localization and host cell invasion/attachment (Liu *et al.* 2014). Ablation of TgCPL, which resides primarily in the PLVAC, resulted in maturation defects of two important microneme proteins, TgM2AP and TgMIC3 (Parussini *et al.* 2010). Disruption of the V-ATPase, which partially localizes to the PLVAC and ELC, resulted in defects in TgMIC3, TgMIC2, and TgM2AP localization, maturation, and secretion (Stasic *et al.* 2019). Rhoptry protein and organelle maturation were also impacted by disruption

of the V-ATPase (Stasic *et al.* 2019). These findings provide additional evidence that the microneme and rhoptry proteins transit through the parasite endolysosomal system where they undergo proteolytic maturation.

TgCPL in the PLVAC was also identified as the maturase for TgCPB (Dou *et al.* 2013), and because both localize to the PLVAC, it is likely that both work together in the degradation of polypeptides in the PLVAC. Although TgCPB was reported to be involved in the maturation of ROP2, 3, and 4 (Que *et al.* 2002), *TgCPB* null mutant parasites exhibit normal maturation of ROP2 (Dou *et al.* 2013). Despite being a highly active protease, TgCPL can limit proteolysis at low concentrations and under suboptimal enzymatic conditions (Parussini *et al.* 2010). That TgCPL is found in low abundance in ELCs, which are likely less acidic, provides a possible explanation for how it contributes to the proteolytic maturation of certain microneme proteins without degrading them further. It should also be noted that a post-Golgi resident protease, TgASP3, is principally responsible for the proteolytic maturation of most microneme and rhoptry proteins (Dogga *et al.* 2017). These studies support a model in which *T. gondii* utilizes endolysosomal proteases for the proteolytic maturation of exocytic proteins, thereby ensuring their efficient processing prior to reaching their respective regulated exocytic organelles.

**Salt homeostasis**

As *T. gondii* advances through its lytic cycle, it encounters sharp changes in the ion composition of its surrounding milieu. Intracellular tachyzoites, which replicate inside a porous parasitophorous vacuole (PV), are surrounded by an ionic environment that is in equilibrium with the host cytosol, in which the concentration of calcium, zinc, chloride, and sodium is low while potassium is high. The concentration of ATP will likely be high while glucose could be low. Upon exit from host cells this surrounding milieu will change to high calcium, zinc, sodium and chloride and low potassium and ATP. Coping with these ionic and nutrient changes is of paramount importance for the extracellular tachyzoite which needs to glide, find a host cell to invade, secrete adhesins needed for attachment and actively invade a targeted cell (Black & Boothroyd 2000; Blader *et al.* 2015). A role for the PLVAC in ion homeostasis was postulated as it was noticed that it existed as a single entity shortly after the parasites egressed and fragmented soon after invasion of host cells (Miranda *et al.* 2010; Parussini *et al.* 2010). Overexpression of TgVP1 resulted in increased tolerance to 150 mM NaCl and significantly more plaques than parental cells under identical conditions (Miranda *et al.* 2010). This result supported a role for the PLVAC in accumulation of sodium and protection of parasite fitness when subjected to salt stress. This interpretation was further supported by the demonstration of proton transport stimulated by $PP_i$ in enriched PLVAC fractions (Miranda *et al.* 2010). The proton gradient generated through the membrane of the PLVAC was collapsed by the addition of sodium, indicating the presence of a sodium/proton exchange activity (Miranda *et al.* 2010). A sodium/proton exchanger TgNHE3 was characterized and localized to the ELC



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and shown to be important for survival of extracellular *T. gondii* in hyperosmotic media. The knock-out mutant of the *TgNHE3* gene was less tolerant to hyperosmotic conditions (Francia *et al.* 2011) and showed significantly lower survival in media with hyperosmotic conditions created with high concentrations of sorbitol (0.75 M), or NaCl (0.5 M), sodium gluconate (0.75 M), or KCl (0.6 M) (Francia *et al.* 2011). A role of the PLVAC in osmotic homeostasis was also shown by the analysis of the TgVP1 knock-out mutant, *Δvp1.* The volume recovery response resulting from exposing *T. gondii* extracellular tachyzoites to hypoosmotic buffers was defective in the *Δvp1* mutant. This deficient response was interpreted as a defective $H^+$-gradient leading to lower capacity to accumulate osmolytes resulting in a defective cell volume response, which also negatively impacted invasion and other stress responses like high extracellular ionic concentrations and hyperosmotic stress. During the process of invasion there is an observable change in cell volume as the parasite squeezes itself through a tight junction that forms between the parasite and the host cell plasma membrane (Mordue *et al*. 1999). Inside the host cell cytosol the parasite appears to recover its normal cell volume, highlighting the importance of cell volume regulation during the lytic cycle of *T. gondii* (Liu *et al.* 2014). Taken together, the experimental evidence supports a role for the PLVAC in ion homeostasis which is also important for controlling osmotic homeostasis in *T. gondii*.

**Calcium homeostasis**

Changes in cytosolic $Ca^{2+}$ are essential for a variety of cellular processes. It has been demonstrated that the endoplasmic reticulum (ER) is the main $Ca^{2+}$ store in most cells. However, the evidence demonstrates that $Ca^{2+}$ can also be stored in acidic compartments. Acidic stores include acidocalcisomes, vacuoles, lysosomes, lysosome-like organelles, secretory granules, and the Golgi apparatus (Patel & Docampo 2010). $Ca^{2+}$ uptake in these compartments is usually mediated by $Ca^{2+}$ pumps or exchangers and for release, acidic stores use two-pore channels or members of the transient receptor potential superfamily (Patel & Docampo 2010). In the case of the PLVAC, a calcium pump TgA1 was shown to localize to a large vacuole interpreted at the time as a large acidocalcisome (Luo *et al.* 2001). A $Ca^{2+}/H^+$ exchange activity was demonstrated in PLVAC-enriched fractions energized with $PP_i$, by the release of protons, taken up by the activity of TgVP1, upon addition of $Ca^{2+}$. The transport of $Ca^{2+}$ by the $Ca^{2+}/H^+$ exchanger uses the energy generated by the proton gradient (Miranda *et al.* 2010). A different experiment with tachyzoites loaded with the $Ca^{2+}$ indicator Fura2 showed that addition of glycyl-L-phenylalanine-naphthylamide (GPN), resulted in an increase in cytosolic $Ca^{2+}$ (Miranda *et al.* 2010). GPN is known to cause osmotic swelling of lysosomes in mammalian cells and $Ca^{2+}$ release into the cytosol, a phenomenon that has been used to demonstrate the presence of $Ca^{2+}$ in lysosomes (Haller *et al*. 1996; Morgan *et al*. 2020). This result indicated that the PLVAC stores $Ca^{2+}$ and could play a role in $Ca^{2+}$ homeostasis or signaling. The V-ATPase would pump protons and contributes to the



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proton gradient through the PLVAC membrane. Conditional knockdown of the V-ATPase resulted in a poorly formed PLVAC and diminished intracellular levels of $Ca^{2+}$ (Stasic *et al.* 2021). Additionally, the release of $Ca^{2+}$ triggered by GPN was significantly reduced in the V-ATPase knockdown mutant (Stasic *et al.* 2021). These data linked proton pumping into the PLVAC to $Ca^{2+}$ storage and cytosolic $Ca^{2+}$ homeostasis. A recent finding of a predicted $Ca^{2+}$ binding protein (TgEFP1) localized to the ELC/PLVAC supports a role for the organelle in $Ca^{2+}$ storage (Dave *et al.* 2022).

The mechanism of $Ca^{2+}$ release from the PLVAC has not been molecularly determined since the initially predicted candidate, the *T. gondii* two-pore channel (TgTPC), was recently localized to the apicoplast (Li *et al*. 2021).

**Storage of toxic components and elements**

An essential role of the plant vacuole is to regulate the concentrations of heavy metal ions like iron, zinc, and copper, within the optimal functional range. Compartmentalization of these ions depends on the activity of the V-$H^+$-ATPase and the V-$H^+$-PPase, which create a proton gradient that is used by a set of tonoplast (plant vacuolar membrane) transporters directly driven by a proton motive force (Sharma *et al*. 2016).

In *T. gondii*, overexpression of the PLVAC-localized TgAQP1 made parasites more resistant to the toxic effect of mercury (Miranda *et al.* 2010). In addition, a zinc transporter (TgZnT) was characterized and localized to the membrane of the PLVAC (Chasen *et al.* 2019). Interestingly both, complete ablation or overexpression of the *TgZnT* gene caused a growth defect in both mutants (Chasen *et al.* 2019). Interestingly the *ΔTgZnT* mutant was hypersensitive to exogenous zinc (Chasen *et al.* 2019). The function of TgZnT was validated by expressing the gene in a yeast strain mutant for its endogenous zinc transporters (Chasen *et al.* 2019). Although TgZnT localized to the PLVAC, it was not clear if zinc is actively transported into the PLVAC. However, these results suggested that *T. gondii* TgZnT is important for the propagation of the parasite and, due to its localization in the PLVAC, it is tempting to speculate that it transports zinc into to the PLVAC. Collectively, these observations demonstrated that the PLVAC may be relevant for protection from environmental ionic stress.

A Vacuolar Iron Transporter (VIT) was recently characterized and found to localize to a vesicular compartment that partially co-localized with TgCPL (Aghabi 2022). The study found that the VIT was important for detoxification of excess iron and for parasite virulence (Aghabi 2022).

**Critical roles for the PLVAC during chronic infection**

The function of the PLVAC in bradyzoites has been mainly studied with respect to its role in protein digestion. Kannan *et al*. (Kannan *et al*. 2021) showed that bradyzoites lacking CPL activity accumulated host-derived mCherry in the PLVAC, indicating that, like tachyzoites, chronic stage *T. gondii* ingests and digests proteins from the cytosol of



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infected cells. Ingestion of host cytosolic mCherry occurred in both *in vitro* differentiated bradyzoites and those derived from brain cysts isolated from infected mice. Although these studies suggest that bradyzoites have an active ingestion pathway, the importance and role of this process during chronic infection remain to be determined.

In addition to digesting host-derived protein, proteolytic digestion in the PLVAC is critical for the degradation of parasite-derived material delivered to the PLVAC via macroautophagy (hereafter referred to as autophagy). Autophagy is a "self-eat" pathway that eukaryotic cells use for several purposes including the turnover of subcellular structures and organelles. Autophagy involves the development and enlargement of double membrane cup termed a phagophore that engulfs cytoplasmic structures in a targeted (selective) or non-specific (bulk) manner. Closure of the phagophore to generate an autophagosome is followed by fusion with an endolysosomal compartment such as lysosomes or a vacuole to create an autolysosome. Earlier work reported autophagosome-like structures in *T. gondii* tachyzoites exposed to resource limited conditions in vitro, suggesting the parasite has a functional autophagy system (Besteiro *et al.* 2011; Ghosh *et al.* 2012). Upon differentiation of bradyzoites lacking TgCPL activity, Di Cristina *et al*. (Di Cristina *et al.* 2017) observed the development of large dark inclusions that were visible by phase contrast microscopy in the cytoplasm of parasites. Analysis by transmission electron microscopy revealed that the dark inclusions contain undigested material including remnants of parasite organelles, consistent with them being autolysosomes with attenuated proteolytic activity. Additional experiments showed the accumulation of TgATG8, a canonical marker of autophagosomes, associated with the PLVAC, indicating defective turnover of autophagosomes. Importantly, CPL-deficient bradyzoites showed a marked loss of viability both *in vitro* and during chronic infection of mice. More recent work showed that bradyzoites lacking the integral membrane protein TgATG9 are defective in autophagy and exhibit a severe loss of viability following *in vitro* differentiation and during chronic infection of mice (Smith *et al*. 2021). Together these studies suggested a critical role for autophagy and the turnover of autophagosomes in bradyzoites, possibly to maintain cellular homeostasis during persistence.

As noted above, TgCRT likely functions to export products of proteolytic digestion (peptides, amino acids) from the PLVAC into the parasite cytoplasm. Consistent with this role, bradyzoites lacking TgCRT showed a striking enlargement of the PLVAC (Kannan *et al.* 2019). The enlargement was partially reversed by inhibition of TgCPL, which is consistent with a proposed role for TgCRT as a transporter of proteolytic products. Interestingly, TgCRT-deficient bradyzoites do not appear to be defective in the turnover of autophagosomal material, suggesting that digestive function is not impaired in such parasites. Other transporters that also function in export of digestive projects probably exist but have yet to be reported.

**Summary and future perspectives and unanswered questions**



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It has been 12 years since the first reports of the presence of the large organelle, termed at the time the PLV or VAC in *T. gondii*. It is interesting that many images of tachyzoites in previous publications clearly showed the PLVAC prominently displayed, but took the discovery in 2010 of PLVAC markers, mainly TgCPL and TgVP1, to identify this organelle. Subsequent publications showing more markers and their roles highlighted the PLVAC lysosomal-like characteristics. The PLVAC is clearly a compartment with multiple functions for *T. gondii* as the plant vacuole is for the plant cell. Some of these functions are more essential than others, which could be related to the conditions used for culturing the parasites or the host cells used. Other functions like aiding in the maturation of important secretory proteins while also acting as the site where proteins are digested is an intriguing biological duality. It is interesting to highlight the greater relevance of some proteins for bradyzoites than tachyzoites. There are likely other functions not currently identified that this organelle plays throughout the parasite lifecycle.

Meanwhile during these 12 years the field of *T. gondii* has expanded significantly due to new genetic tools like CRISPR/Cas9 for gene editing, use of biotin-ligase fusion-proteins to identify proximal and interacting proteins with common subcellular localizations, and the adaptation of tools to regulate expression transcriptionally or translationally, enabling studies of more immediate phenotypic differences without the risk of compensatory changes. The availability of CRISPR/Cas9 tools resulted in a burst of interest in the characterization of the role of molecules in the slow-growing bradyzoite, which has resulted in several important discoveries. We anticipate more proteins of the PLVAC will be identified by these revolutionary technologies in the coming years. Some of these could help understand its interaction with other endolysosomal compartments, its fast biogenesis in the extracellular parasite and its fragmentation during intracellular replication, and why the PLVAC with highly lytic activities aids in the maturation of secretory proteins.

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**Figure Legend:**

Schematic of a *T. gondii* tachyzoite highlighting the endolysosomal organelles, ELC and PLVAC and their connection with the secretory organelles (rhoptries, micronemes and dense granules). The inset shows an enlarged PLVAC with the known components. Some activities are shown with dashed lines as they have not been molecularly demonstrated. TgVP1, vacuolar proton pyrophosphatase; TgVATPase, vacuolar proton transport ATPase; NHE3, sodium proton exchanger; TgZnT, zinc transporter, TgCRT, chloroquine resistance transporter; TgPiT, phosphate sodium transporter; TgAQP1, aquaporin; TgA1, $Ca^{2+}$-ATPase; $Ca^{2+}/H^{+}$, $Ca^{2+}$ proton exchanger; TgCPL, cathepsin L; TgCPB, cathepsin B; TgCPC1, cathepsin C1; TgASP1, aspartic protease.

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**Table 1: Molecular Components of the PLVAC**

| Protein Name/gene ID | Known Function | Localization | Membrane/ luminal | Phenotype (Fitness Score) | Re |
|---|---|---|---|---|---|
| **Cathepsin Protease L (TGME49_321530)** | Cysteine Protease | PLVAC | Luminal | Dispensable in tachyzoites (0.68)/Significant in bradyzoites | (Paru 2010 Carru |
| **Cathepsin Protease B (TGME49_249670)** | Cysteine protease | PLVAC | Luminal | Dispensable (0.99) | (Que Dou a Carru |
| **Aspartyl Protease 1 (TGME49_201840)** | Aspartic Peptidase family | PLVAC | Luminal | Dispensable (-0.36) | (McD 2020 |
| **Vacuolar Pyrophosphatase (TGME49_248670)** | V-type $H^+$-translocating pyrophosphatase VP1 | PLVAC, PM, LE compartment, acidocalcisomes | Membrane | Dispensable (1.27) | (Mira 2010 2014 |
| **Aquaporin1 (TGME49_215450)** | Aquaporin water channel | PLVAC, PM | Membrane | Dispensable (0.84) | (Mira 2010 |
| **Chloroquine Resistance Transporter (TGME49_313930)** | CRT-like Chloroquine resistance transporter like | PLVAC | Membrane | Mild in tachyzoites/Moderate in bradyzoites (0.96) | (War 2014 al. 20 (Thor 2019 |
| **NHE3 (TGME49_305180)** | $Na^+/H^+$ Exchanger Cation/$H^+$ exchanger | PLVAC, LE | Membrane | Dispensable (0.0) | (Fran 2011 |
| **V-$H^+$-ATPase (multiple gene IDs)** | Vacuolar-$H^+$-ATPase Multi-subunit | PLVAC, PM, pre-rhoptries | Membrane | Essential | (Stas 2019 |
| **Zinc Transporter (TgZnT) (TGME49_251630)** | Vacuolar Zinc Transporter | PLVAC, acidocalcisomes | Membrane | Growth defect of mutants (-3.06) | (Chas 2019 |
| **Pi-$Na^+$ Transporter (TGME49_240210)** | Phosphate transporter, PiT family | PLVAC, cytoplasmic vesicles, PM | Membrane | Mild growth defect of mutants. Lower virulence (-1.32) | (Asad 2020 |
| **Vacuolar Iron Transporter (TgVIT) (TGME49_266800)** | Vacuolar Iron Transporter | PLVAC, other unknown vesicles | Membrane | Mild growth defect of mutants. Lower virulence (-1.22) | (Agha |

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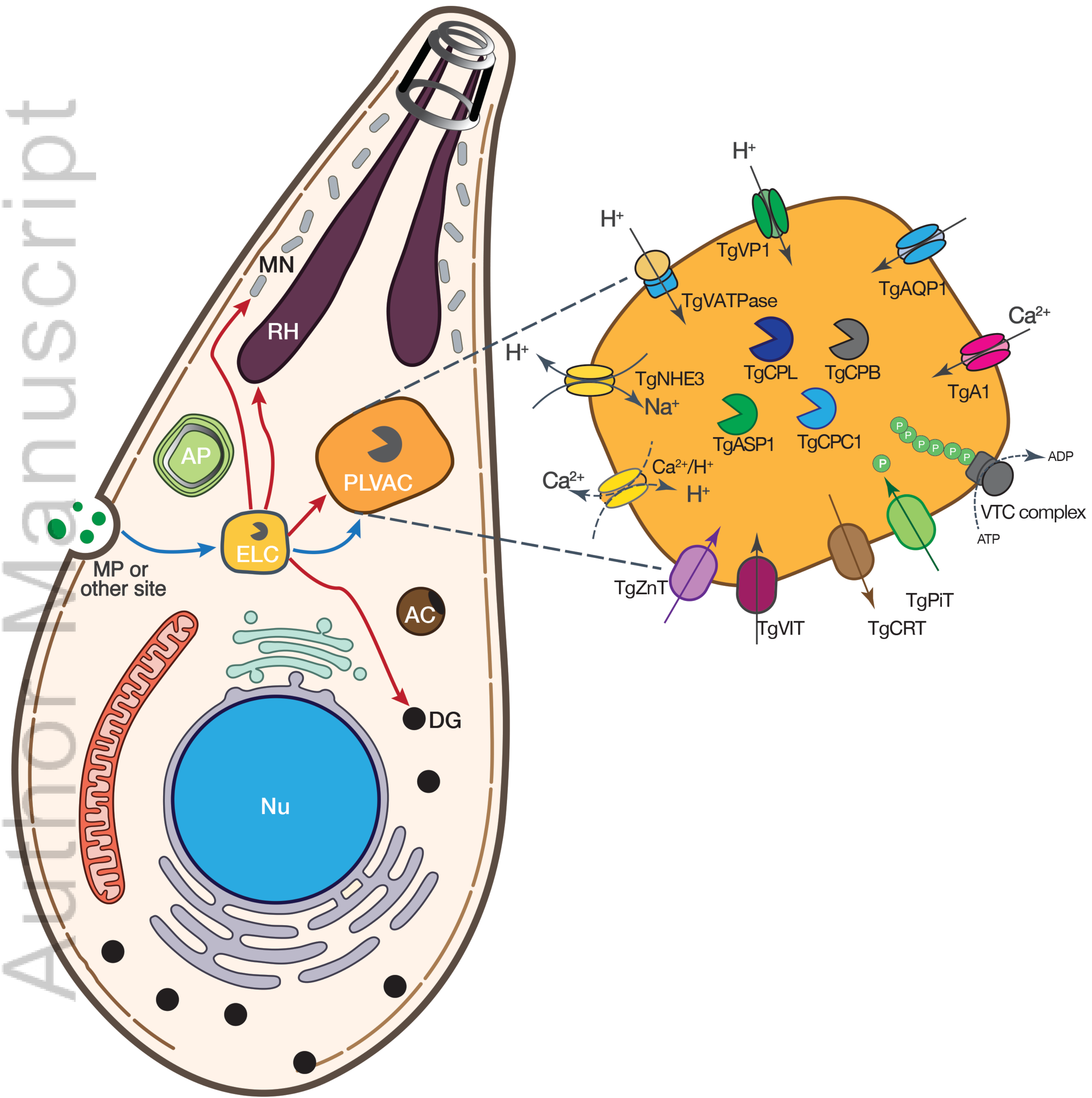


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