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Dense granule biogenesis, secretion and function in *Toxoplasma gondii*

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Abstract

Toxoplasma gondii is an obligate intracellular parasite and the causative agent of Toxoplasmosis. A key to understanding and treating the disease lies with determining how the parasite can survive and replicate within cells of its host. Proteins released from specialized secretory vesicles, named the dense granules (DGs), have diverse functions that are critical for adapting the intracellular environment, and are thus key to survival and pathogenicity. In this review, we describe the current understanding and outstanding questions regarding dense granule biogenesis, trafficking, and regulation of secretion. In addition, we provide an overview of dense granule protein ("GRA") function upon secretion, with a focus on proteins that have recently been identified.

Keywords

Toxoplasma gondii ; dense granule; host-pathogen interactions; vesicle secretion

THE Apicomplexan phylum consists of a diverse collection of obligate intracellular parasites of significant medical and veterinary importance. Apicomplexa include *Plasmodium spp.*, the causative agent of malaria that is responsible for approximately half a million deaths per year (WHO Malaria report 2020), *Cryptosporidium* which can cause life-threatening diarrheal disease (Khalil et al. 2018), and *Toxoplasma gondii,* the focus of this review and the causative agent of toxoplasmosis (Weiss and Dubey 2009). Parasites in this phylum have diverse and complex lifecycles but the intracellular portion of their lifecycle usually involves replication within a specialized vacuole called the parasitophorous vacuole (PV) (Aly et al. 2009; Blader et al. 2015; Francia and Striepen 2014).

In *T. gondii*, three types of secretory vesicles play vital roles in parasite invasion into the host cell, replication within the PV, and parasite egress. Micronemes are small, elongated vesicles located predominately at the parasite's apical end. They are secreted in a calcium dependent manner and are vital for motility of extracellular parasites, host cell invasion and egress (Bisio and Soldati-Favre 2019; Lourido et al. 2012; Lourido et al. 2010; Paredes-

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Santos et al. 2012). Rhoptries are club-shaped organelles containing proteins necessary for invasion and modification of host cells, including a number of important virulence factors (Bradley and Sibley 2007). Secretion of rhoptry proteins is tightly controlled and only triggered after strong attachment to the host cell (Alexander et al. 2006; Sparvoli and Lebrun 2021). Dense granules (DG) were first described in *Toxoplasma* in 1968 (Sheffield and Melton 1968). These 200nm membrane enclosed vesicles have an electron dense crystalline protein core and are vital for intracellular survival.

During invasion, the PV is formed from host plasma membrane (PM). Host proteins are removed from the newly formed PV membrane (PVM) which presents a physical boundary between the parasite and host cell cytosol (Mordue et al. 1999; Schwab et al. 1994). Proteins released from the dense granules immediately after invasion are vital for modifying the PV and intracellular environment of the host. Understanding the function of dense granule proteins (collectively termed “GRA” proteins) and the mechanisms by which dense granules are synthesized, transported, and triggered for secretion is fundamental for understanding infection.

Dense Granule biogenesis, transport, and secretion

GRA proteins are synthesized in the ER and proteolytically processed in the Golgi

The majority of GRA proteins, like the proteins destined for the micronemes and rhoptries, contain an N-terminal ER-targeting signal peptide, and thus enter the secretory pathway via synthesis and translocation at the rough endoplasmic reticulum (Table S1). As in other eukaryotes, the signal peptide is thought to be removed co-translationally by a signal peptidase, however the function of a putative signal peptidase in *T. gondii* (TgME49_280740) has not been verified experimentally.

Many dense granule proteins contain a single transmembrane (TM) domain (Table S1). In other eukaryotes, proteins containing both a signal peptide and a TM domain are inserted into the ER membrane during synthesis and subsequently trafficked to either the plasma membrane or the membrane of another compartment within the endomembrane system. Expression of the TM-domain containing protein GRA5 in mammalian cells resulted in the protein trafficking to the plasma membrane (Gendrin et al. 2008). In parasites, GRA5 and other TM domain-containing GRA proteins are translocated across the ER membrane into the lumen and trafficked through the endomembrane system, which involves export from the ER, trafficking through the Golgi to dense granules, and finally secreted from the parasite (Fig. 1) (Braun et al. 2008; Gendrin et al. 2008; Lecordier et al. 1999; Mercier et al. 2005). Thus, there must be parasite specific mechanisms to facilitate GRA protein trafficking to the ER lumen and ensure GRA proteins are not co-translationally inserted into the ER membrane. GRA proteins form higher ordered aggregates that may mask the TM domains preventing premature membrane insertion (Braun et al. 2008) and a recently identified chaperone (GRA45) also appears to play a role.

GRA45 has structural similarity to the alpha crystalline domain (ACD) small heat shock proteins and may aid the trafficking of other GRA proteins through the secretory pathway (Wang et al. 2020). When key residues in the ACD domain of GRA45 were mutated,

trafficking of GRA7 through *T. gondii's* endomembrane system was retarded and aberrant GRA protein aggregates were formed. In addition, in GRA45 knockout parasites, a number of GRA proteins that normally associate with PVM were retained in the PV lumen which would indicate misfolding or aberrant protein-protein interactions and perturbed membrane insertion (Wang et al. 2020). Thus, it is likely a combination of aggregation and chaperone binding that masks the TM domains and prevents their insertion into membranes within the secretory pathway (Braun et al. 2008; Gendrin et al. 2008; Wang et al. 2020).

Once synthesis and translocation into the ER lumen is complete, proteins are trafficked between organelles in the endomembrane pathway via transport vesicles. Conserved, membrane-associated protein complexes drive vesicle budding from the donor compartment, vesicle transport and then tethering, docking and membrane fusion with the target membrane. SNARE complexes control vesicle docking and membrane fusion at the target membrane, and are composed of four coiled-coil domain containing proteins: Three Q SNAREs on the target membrane, designated Qa, Qb and Qc according to their position in the complex and one vesicle associated R-SNARE (Fasshauer et al. 1998; Sauvola and Littleton 2021). In *T. gondii*, a SNARE complex at the cis-Golgi composed of syntaxin 5 (TgStx5: Qa), TgGS27 (Qb), TgBet1 (Qc), and TgSec22b (R) control ER to Golgi anterograde trafficking (Cao et al. 2021) (Fig. 2). TgGS27 knockdown parasites exhibited impaired formation of the micronemes and rhoptries, and although the trafficking of proteins to the dense granules was not addressed directly in this study, it is probable that DG trafficking would also be impacted by the loss of this SNARE complex (Cao et al. 2021).

SNARE complex assembly and function is regulated by the Sec1/Munc18 family of proteins (Wang et al. 2017). Depletion of the Golgi localized Sec1/Munc18 family protein, SLY1, impairs the trafficking of proteins destined for all three secretory vesicles, most likely by regulating ER to Golgi trafficking. Although a direct interaction between SLY1 and the TgGS27 SNARE complex has not been demonstrated (Bisio et al. 2020; Cao et al. 2021; Demircioglu et al. 2014; Ossig et al. 1991) (Fig. 2).

DG formation at the TGN

Upon arrival in the Golgi, GRA proteins containing a TEXEL motif (*Toxoplasma* export motif) with the amino acids RRLxxx are further proteolytically processed by the aspartyl protease, ASP5 (Table S1) (Coffey et al. 2015; Coffey et al. 2016; Coffey et al. 2018; Curt-Varesano et al. 2016; Hammoudi et al. 2015). In ASP5 knockout parasites, a number GRA proteins, the functions of which will be discussed in depth below, were mislocalized upon release from the parasite. Specifically, GRA16 failed to be exported from the PV (Coffey et al. 2015; Curt-Varesano et al. 2016) and GRA44, MYR1 and MAF1 failed to localize to the PVM (Coffey et al. 2015; Coffey et al. 2018) indicating that loss of ASP5 severely impacts the parasite's ability to modulate the intracellular environment of the host cell and control host signaling pathways, resulting in a decreased parasite fitness (Coffey et al. 2015; Hammoudi et al. 2015). Surprisingly, mutation of TEXEL motifs in MYR1 and GRA44 did not affect protein insertion into the PV or protein function (Blakely et al. 2020; Cygan et al. 2021). One interpretation of this conflicting data is that proteolysis is critical for the function of some but not all GRA's cleaved by ASP5. For example, if the function

of a molecular chaperone such as GRA45 is perturbed by loss of ASP5, then this could lead to the secretion of mis-folded or aggregated GRA proteins whose activity is GRA45 dependent. In further support of this idea, translocation of GRA24 across the PV membrane was dependent on ASP5 even though it does not contain a TEXEL motif and is not cleaved by the protease (Coffey et al. 2015; Curt-Varesano et al. 2016; Hammoudi et al. 2015).

Mature dense granules bud directly from the trans-Golgi network (TGN) and are morphologically similar to dense core vesicles in neuroendocrine cells that are part of the regulated secretory pathway in mammalian cells. Despite these similarities, dense granule formation and trafficking has aspects of both the constitutive and regulated secretory pathways (CSP and RSP, respectively) (Fig. 2) (reviewed by Gondré-Lewis et al. 2012). Briefly, vesicles in the CSP in mammalian cells are formed at the medial and trans-Golgi in a clathrin independent manner, are electron lucent and ~100nm in size (Orci et al. 1987; Walworth and Novick 1987). There is no specific sorting signal, and these vesicles serve as the “default” pathway for secretion. Constitutive secretory vesicles (CSVs) have no storage pool and are continually secreted; thus, the rate of secretion is dependent on the rate of synthesis. It is estimated that the total time from formation to secretion is approximately 30 minutes (Gondré-Lewis et al. 2012). In contrast, vesicles in the RSP in mammalian cells form initially as immature vesicles that bud from the TGN in a clathrin dependent manner. Removal of mistargeted proteins, and vesicle maturation involving acidification of the vesicle and crystallization of protein contents, occurs before secretion. Pools of mature secretory vesicles are retained in the cell cortex until a secretagogue (usually increased intracellular calcium) triggers vesicle fusion with the plasma membrane (Gondré-Lewis et al. 2012; Park and Loh 2008; Verhage and Sørensen 2008; Wang and Hsu 2006).

Similar to RSP’s, DG formation at the TGN is clathrin-dependent (Pieperhoff et al. 2013). *T. gondii* has a single clathrin heavy chain (CHC) that is localized predominately at the TGN. Expression of a dominant negative CHC fragment leads to accumulation of a DG marker in the ER and Golgi, as well as disrupting trafficking to the rhoptries and micronemes indicating that CHC is required for vesicle formation at the TGN. However, deletion of the clathrin adaptor AP1 that links the vesicle membrane to the clathrin cage, resulted in mis-sorting of microneme and rhoptry proteins but was not required for formation of the dense granules (Ngô et al. 2003; Venugopal et al. 2017). Thus, the adaptors targeting CHC to dense granules budding from the TGN have not yet been identified. The direct budding of DGs from the TGN is distinct from the more complex trafficking pathway of proteins destined for the micronemes and rhoptries, which traffic through one or more post-Golgi compartments before delivery to mature rhoptry and microneme compartments (reviewed by Venugopal and Marion 2018).

Like vesicles in the CSP, numerous lines of evidence indicate that dense granules appear to be the default pathway for secretory protein trafficking. Removal of the GPI anchor signal sequence from the surface protein SAG1 results in mis-targeting to the dense granules (Heaslip et al. 2016; Striepen et al. 1998). While expression of *E. coli* alkaline phosphatase and β-lactamase with an N-terminal signal peptide results in trafficking to the dense granules and secretion into the PV lumen (Karsten et al. 1998). Similarly, when the first 68 amino acids of the microneme protein MIC3 were tagged with GFP, MIC3 was directed to the

dense granules (Striepen et al. 2001). Thus, unlike rhoptry proteins which must contain a dileucine (LL) motif or a tyrosine-based sorting signal (Yxxϕ motif), there appears to be no requisite sequence for trafficking to the dense granules (Hoppe et al. 2000; Ngô et al. 2003)

Proteins in the RSP are sorted at the TGN by aggregation that is induced by low pH, calcium, or zinc as demonstrated by electron microscopy and pulse chase experiments. After sorting, the vesicles are acidified further by proton pumps on the vesicle membrane (Chanat and Huttner 1991; Germanos et al. 2021; Gondré-Lewis et al. 2012). These protein aggregates are responsible for the electron dense appearance of granules in electron microscopy images, for which this class of vesicles is named (i.e., dense granules or large dense core vesicles (DG or LDCV)). GRA proteins do form electron dense aggregates in *T. gondii* (Labruyere et al. 1999; Wang et al. 2020) but it is not understood when along the secretory pathway this occurs, or what environmental conditions within the granule lumen facilitate aggregate formation. The pH of the dense granule lumen has not been investigated thoroughly. DAMP (3-(2,4-dinitroanilino)-3'amino-N-methyldipropylamine), which accumulates in acidic cellular compartments, only labeled the rhoptries, suggesting that the dense granules were not acidic (Shaw et al. 1998), however, this question warrants further investigation. Outstanding questions include: What is the pH of the DG lumen and what is the concentration of calcium and zinc? If the DG are acidic, what proton pumps are responsible for acidification? Does acidification or calcium and zinc ions play a role in protein aggregation?

Mechanisms of dense granule transport

In many cell types, secretory vesicles are trafficked from the Golgi to the plasma membrane in an active, motor-driven manner. In mammalian cells, kinesin motion on microtubule tracks controls anterograde vesicle motion (Hirokawa et al. 2009), while in budding and fission yeast, vesicle transport is driven by myosin V movement on actin tracks (Hammer and Sellers 2011). Live cell imaging of GFP labeled dense granules demonstrated that DG movements are highly dynamic and DGs exhibit long directed, motor driven movements predominately along the parasite periphery. Depolymerization of F-actin with cytochalasin D or conditional knockdown of an unconventional myosin motor, MyoF, significantly reduced directed vesicle movement. Depolymerization of parasite microtubules had no effect on directed movement indicating dense granule motion is an acto-myosin dependent process (Heaslip et al. 2016). Interestingly, dense granule secretion is not affected by the loss of MyoF, suggesting that this protein is exclusively involved in vesicle transport and is not required for the secretion event itself. One surprising finding that emerged from the analysis of dense granule movements was that granules do not appear to be tethered at or near plasma membrane release sites, on the contrary vesicle movement along the periphery appeared almost random with movements being bidirectionally towards both the apical and basal ends of the parasite. Frequent direction changes were observed (Heaslip et al. 2016), suggesting that the purpose of vesicle movement was to increase the chances of a granule encountering a granule release site.

In order to control the motion of membrane bound cargo, transport motors typically bind to adaptor proteins on the surface of a vesicle/organelle with their C-terminal cargo

binding domains. Diffraction limited images of fluorescently labeled MyoF and dense granules indicate that MyoF is not enriched on the granule surface (Heaslip et al. 2016). Moreover, no MyoF tail binding proteins have been identified to date, and so it is not clear if MyoF mediates dense granule transport through a direct interaction with the granule surface. Elucidating MyoFs mechanism of action is complicated by fact that MyoF is also required for apicoplast (a non-photosynthetic plasmid organelle) inheritance, transport of Rab6 vesicles, movement of ER tubules and positioning of the post-Golgi compartments (Carmeille et al. 2021). Future studies focused on identifying MyoF interacting proteins are crucial for a complete understanding of the mechanisms of dense granule movement.

Dense Granule protein secretion

There are many outstanding questions in regards to the mechanism, regulation and location of GRA protein secretion.

Is GRA protein secretion regulated or constitutive?—As discussed in detail above, DG biogenesis has components of both the CSP and RSP, and whether DG secretion is regulated or constitutive has been the subject of debate in the literature (Chaturvedi et al. 1999; Coppens et al. 1999). Numerous lines of evidence support the idea that DG secretion is regulated. First, secretion from DGs was not affected by treatment with brefeldin A, which inhibits trafficking between the ER and Golgi and results in decreased secretion from CSVs in mammalian cells (Coppens et al. 1999). DG release was originally thought to be independent of calcium. However, it has recently been shown that the dense granule secretion is negatively regulated by calcium (Katris et al. 2019). Secretion is also triggered by incubation in bovine serum albumin (BSA) or fetal calf serum (FCS) so it is possible there is a yet unidentified, secretagogue which induces secretion from DGs (Coppens et al. 1999).

GRA proteins accumulate in the PV within minutes of invasion and it has been proposed that this rapid accumulation represents an upregulation or burst of secretion immediately after invasion (Carruthers and Sibley 1997; Dubremetz et al. 1993; Sibley et al. 1995). However, GRAs are also secreted from extracellular parasites, and a direct comparison of the DG secretion rates from extracellular, newly invaded, and replicating parasites has not yet been achieved. Changes in the rate of secretion in different cellular environments would provide evidence for regulated secretion.

How do DG traverse the IMC to reach the plasma membrane?—Despite extensive imaging of intracellular *T. gondii* by electron microscopy, there are only a few images which appear to depict dense granule secretion events, probably due to the quick and transient nature of this cellular process (Dubremetz et al. 1993; Paredes-Santos et al. 2013). These images show dense granule secretion occurring via direct fusion with the parasite plasma membrane through gaps in the inner membrane complex (IMC). Intriguingly, where the "gaps" in the IMC are located is not understood. Large gaps in the IMC at the apical and basal ends are plugged by the apical and basal complexes respectively (Dos Santos Pacheco et al. 2020; Morano and Dvorin 2021) (Fig. 1), and are not thought to be involved in DG secretion. In addition, the two other characterized gaps in the IMC are the apical annuli

and the micropore. The parasite contains a single micropore, an invagination in the PM thought to be the site of endocytosis rather than secretion (Nichols et al. 1994). Each parasite also contains 5–6 apical annuli, ring shaped structures with a diameter between 200–400nm and located ~1.5μm from the apical complex on the lateral sides of the parasite (Engelberg et al. 2020; Hu et al. 2006) (Fig. 1). The imaging that exists indicate that dense granule secretion may occur from the apical lateral sides of the parasite at a location consistent with positioning of the apical annuli (Dubremetz et al. 1993; Paredes-Santos et al. 2013; Sibley et al. 1995). However, there has been no direct evidence that secretion of the dense granules occurs at this position.

Few proteins have been identified that are essential for dense granule secretion. The small GTPase Rab11a is found on a subset of DGs and is required for both dense granule transport and secretion (Venugopal et al. 2020), although the mechanism by which Rab11a controls secretion has not been elucidated. Only one other protein is known to localize in the DG membrane, GRA46, and the function of this protein has not been defined (Coffey et al. 2018). Thus, additional studies are needed to generate a catalog of DG membrane-associated proteins whose functions in transport and secretion could then be investigated.

Live cell imaging is one possible methodology that could be utilized to address many of the outstanding questions in regard to dense granule secretion. Individual DG secretion events at the parasite PM have never been visualized. One major limitation to imaging dense granule secretion events is the accumulation of secreted fluorophore in the PV upon secretion making imaging of any subsequent secretion events challenging. Overcoming this technical limitation will enable the identification of the DG secretion site, quantification of GRA secretion rates, and characterization of proteins important for this cellular process.

Functions of Dense Granule (GRA) Proteins

GRA proteins play pivotal roles in mediating host-parasite interactions. Upon secretion, GRA proteins will either remain in the PV lumen, interact with the intravacuolar network (IVN, described below), be inserted into the PVM, or secreted out of the PVM into the host cell. The PV is only permeable to molecules less than ~1kDa (Schwab et al. 1994), therefore the ionic composition and pH of the cytosol and PV space are likely equivalent but the parasite is not in direct contact with the vast majority of cytosolic proteins or organelles. Thus, the PV is the interface for many host-parasite interactions. Over 60 GRA proteins have been identified to date, first through the generation of antibodies against the parasite lysates or secreted antigens (Carey et al. 2000; Cesbron-delauw et al. 1989; Leriche and Dubremetz 1991), and more recently from BioID proteomics and CRISPR-Cas9 functional characterization studies (Table S1) (Bai et al. 2018; Cygan et al. 2021; Nadipuram et al. 2016). Only a fraction of these proteins have a well characterized role in parasite infection. In this section we will focus on discussing new insights into GRA protein function and describe recently identified GRA proteins. A comprehensive description of all GRA proteins is not possible due to space constrains but we refer the reader to a number of other excellent reviews (Clough and Frickel 2017; Panas and Boothroyd 2021;Saeij and Frickel 2017;Mercier and Cesbron-Delauw 2015) .

Nutrient acquisition

Morphology and Biogenesis of the IVN: The PVM is a physical barrier between the host cell and parasite that must be overcome for the parasite to scavenge nutrients and lipids from the host. GRA proteins induce a number of dramatic alterations to the PVM that are vital for nutrient acquisition. The intravacuolar network (IVN) is an extensive membranous tubular network that at times is continuous with the PVM (Coppens et al. 2000;Sibley et al. 1995) (Fig. 1). Newly invaded parasites contain an ~400nm intracellular compartment filled with membranous tubules that could be a source of lipids for initial IVN formation (Romano et al. 2017). This compartment was only recently seen in electron microscope images and no biomarkers for this compartment have been identified leaving many outstanding questions regarding the biogenesis, regulation, and mechanisms of IVN tubule secretion. The IVN forms within minutes of invasion with tubular membranes emanating from the parasites' basal end (Sibley et al. 1995). Shortly thereafter, dense granule proteins including GRA2, GRA4, GRA6, GRA9, and GRA12 associate with the IVN (Cesbron-Delauw et al. 2008; Mercier et al. 2002; Rommereim et al. 2016; Travier et al. 2008). Deletion of IVN associated GRA4, GRA9 and GRA12 did not lead to any apparent alterations in the IVN structure, while GRA2 and GRA6 knockout parasites exhibit defects in IVN morphology (Cesbron-Delauw et al. 2008; Mercier et al. 2002; Travier et al. 2008).The IVN expands as parasites replicate, and while the parasite may contribute some lipids for initial formation of the IVN, the host is the major source of lipids for IVN expansion (Caffaro and Boothroyd 2011). PtdSer decarboxylase (TgPSD1) is a protein that is secreted by dense granules and localizes in the PV (Gupta et al. 2012). TgPSD1 decarboxylates phosphatidylserine (PtdSer) to create phosphatidylethanolamine (PtdEtn), an important building block for membrane biogenesis. While it has not been directly observed to aid in expansion of the parasite plasma membrane or the PV membrane, it does have the capacity for carboxylase activity on membranes (Gupta et al. 2012).

Lipid acquisition: *T. gondii* scavenges a wide variety of lipids from the host cell. Sphingolipids are acquired by engulfing a variety of host-Golgi derived Rab-associated vesicles (Romano et al. 2013; Romano et al. 2017). Intact vesicles are concentrated within the IVN and vesicle uptake is reduced in GRA2 and GRA6 double knockout parasites. Until recently, it was unclear how vesicles traversed the PVM. A recent study using both light and electron microscopy revealed double membraned vesicular structures in the PV lumen, indicating that the host derived vesicles become surrounded with PVM before internal budding into the PVM lumen (Romano et al. 2017). The association with yet another IVN associated dense granule protein, TgLCAT (a lecithin-cholesterol acyltransferase), with internalized vesicles suggests this protein plays a role in destabilization of the PV derived membrane surrounding the Rab vesicles before uptake into the parasite (Pszenny et al. 2016; Romano et al. 2017).

T. gondii is an auxotroph for cholesterol (Blader and Koshy 2014; Coppens et al. 2000). This vital lipid is acquired from host endosomal and lysosomal vesicles using PVM tubules distinct from the IVN (Fig. 1) (Coppens et al. 2000; Coppens et al. 2006). These tubule invaginations, termed "Host Organelle-Sequestering Tubulo-structures" (HOST) are ~100nm in diameter. The lumen is continuous with the host cytosol and contains host microtubules

that may act as a track for vesicle transport to the HOST tip. The HOSTs contain transverse striations in TEM images indicating proteinaceous collars with potential roles in tubule formation, stability, or constriction (Coppens et al. 2006). Lysosomal vesicles are also found in the PV, surrounded by PV-derived membrane, so the proposed mechanism of uptake is similar to what is described above whereby vesicles undergo internal budding into the PV lumen (Coppens et al. 2006).

It has been proposed that GRA7 is a component of the proteinaceous collars associated with the HOSTs. Recombinant GRA7 can tubulate liposomes *in vitro* and GRA7 knockouts exhibit decreased growth rates in nutrient depleted media. However, it was not directly determined if loss of GRA7 affected HOST formation (Coppens et al. 2006). GRA7 along with GRA3 and GRA14 are found in filamentous extensions (termed PVM projections, PVMPs) which extend into the host cytosol (Dubremetz et al. 1993; Dunn et al. 2020). PVMPs may also play a role in vesicle or organelle association with the PVM although their function is poorly defined.

Another major source of lipids for the parasite comes from host neutral lipid droplets (LD) (Hu et al. 2017; Nolan et al. 2017). Infected cells have more lipid droplets compared to uninfected cells (Hu et al. 2017; Nolan et al. 2017). This increase in lipid droplet production is dependent on an unidentified parasite factor secreted into the host (Hu et al. 2017). Host LDs are found both surrounding the PV and within the PV lumen, and neutral lipids from the host are incorporated into organellar membranes of the parasite. As was the case with other vesicle types described above loss of GRA2, GRA6 and LCAT resulted in decreased LD uptake (Nolan et al. 2017).

Acquisition of soluble proteins from the host cytosol: In 2014 Dou and colleagues (Dou et al. 2014) demonstrated that parasites can acquire soluble proteins from the host, as evidenced by the uptake in fluorescent proteins from the host cytosol into the parasite. Reductions in host protein uptake were seen in GRA2 and GRA6 knockout parasites, again implicating the IVN in this process (Dou et al. 2014; Rommereim et al. 2016). Expression of a dominant negative variant of VASP4A, a component of the host ESCRT (Endosomal Sorting Complex Required for Transport) decreased protein uptake to the parasite. GRA14 and GRA64, two single TM domain containing dense granule proteins, bind ESCRT components, and while loss of GRA14 resulted in reduced host protein uptake, loss of GRA64 had no effect (Mayoral., unpubl. data BioRxiv: 2021.11.02.467042; Rivera-Cuevas et al. 2021). The connection, if any, between ESCRT mediated uptake of host material and other uptake mechanisms at the PVM/IVN, should be explored to understand this process in more depth..

Diffusion between PV lumen and cytosol: The PV is only permeable to molecules less than ~1kDa (Schwab et al. 1994). Two proteins, GRA17 and GRA23, form pores within the PVM which allow for the diffusion of small molecules between the host cytosol and PV lumen (Schwab et al. 1994; Gold et al. 2015). Loss of GRA17 led to swelling of PV, possibly due to osmolarity differences between the PV lumen and cytosol. In addition, GRA17 knockout parasites exhibited growth defects that could be attributed either to

nutrient deprivation or inability of the parasite to remove toxic byproducts from the PV (Gold et al. 2015).

Host organelles and cytoskeleton associate with the PV

Host Mitochondria association (HMA): Host mitochondria association (HMA) has been extensively studied (Blank et al. 2021; Crawford et al. 2006; Jones et al. 1972; Li et al. 2022; Pernas et al. 2014) Mitochondria that associate with the PV have an approximately 3-fold increase in surface area compared to cytosolic mitochondria from infected cells due to enhanced mitochondrial fusion mediated by the outer mitochondrial membrane (OMM) proteins mitofusin 1 (Mfn1) and mitofusin 2 (Mfn2) (Pernas et al. 2014; Pernas et al. 2018). Mitochondria play a role in host defenses against infection by increasing the uptake of fatty acids (FA) and thereby reducing the pool of FA available to the parasite (Pernas et al. 2018), a process that is also dependent on Mfn1 and Mfn2 (Li et al. 2022; Pernas et al. 2018).

Not all strains of *T. gondii* display HMA; type I and III strains exhibit HMA while type II stains do not (Pernas et al. 2014). By analyzing the differential gene expression between the three strains, Pernas and colleagues identified the dense granule protein Mitochondria Association Factor 1 (MAF1) as necessary and sufficient for HMA (Blank et al. 2021; Pernas et al. 2014). MAF1 contains a transmembrane domain and is inserted into the PVM. The C-terminus exposed to the host cytosol binds (either directly or indirectly) the OMM protein TOM70 (Blank et al. 2021; Li et al. 2022). HMA induces shedding of the OMM and decreases expression of Mfn1 and Mfn2 thereby counteracting the host defenses designed to limit parasite uptake of FA (Blank et al. 2021; Li et al. 2022). In addition, infection with HMA+ strains have altered cytokine production compared to HMA- strains. Surprisingly, expression of MAF1 in the MAF1-negative type II strain did not alter parasite growth in culture or parasite burden in mice (Pernas et al. 2014).

Host Golgi and ER association with the PV: In addition to mitochondria, host ER and fragmented Golgi stacks associate with PV periphery (Coppens et al. 2000; Romano et al. 2013; Sinai et al. 1997). GRA3 associates with Golgi purified from CHO cells, and also binds phosphatidylinositol lipids via its C-terminus (Deffieu et al. 2019). GRA3 knockouts display reduced entry of host Golgi vesicles into the PV supporting a potential role in lipid scavenging from the host (Deffieu et al. 2019), whereas recruitment of Golgi stacks to the PV was not perturbed. GRA3 knockout parasites exhibited no growth defects in cell culture but exhibited attenuated virulence *in vivo* (Craver and Knoll 2007).

Host cytoskeleton recruitment: The PV is frequently located adjacent to the host nucleus and centrosome and becomes encased in host microtubules and vimentin intermediate filaments (Halonen and Weidner 1994; Walker et al. 2008). Vimentin may play a beneficial role in retaining the PV in a host perinuclear region (Halonen and Weidner 1994), and by extension in closer proximity to host perinuclear organelles (ER, MTOC, Golgi). Bundling of host microtubules around the PV may provide a highway for organelle trafficking to the PV by microtubule associated motor protein. The mechanism by which microtubules and vimentin associate with the PV is not known. However, given the role of GRA proteins in organelle recruitment, it is possible that as yet unidentified GRA proteins

also mediate the recruitment of cytoskeletal elements to the PV. Further work will be required to determine the mechanism and function of the PV-host cytoskeletal association.

Mechanism of GRA protein export from the PV

A number of dense granule proteins are exported out of the PV into the host cell cytosol and nucleus, including GRA16 (Bougdour et al. 2013), GRA24 (Braun et al. 2013), IST1 (Gay et al. 2016), GRA18 (He et al. 2018), and TEEGR/HCE1 (Braun et al. 2019; Panas et al. 2019). As described in more detail below, these proteins play a variety of roles in regulating host cell gene expression and immune response pathways. MYR1, MYR2 and MYR3 (short for c-Myc regulation), were identified in a mutagenesis screen designed to identify the effector responsible for inducing upregulation of host c-myc (Franco et al. 2016; Marino et al. 2018). As it turned out, these proteins were not the direct effectors of c-myc induction but components of the translocon that controls export of the c-myc effector and other GRA proteins to the host cytosol. All three proteins contain TM domains and are localized to the PVM. MYR1 is cleaved by the protease ASP5 (described in more detail above in the section entitled “DG formation at the TGN) before secretion and both fragments interact directly with MYR3 (Marino et al. 2018). Four additional components of the translocon, GRA44, GRA45, PPM3C, ROP17 and MYR4, were subsequently identified by immunoprecipitation (Blakely et al. 2020; Cygan et al. 2020; Mayoral et al. 2020; Panas et al. 2019; Wang et al. 2020). Knockout of all MYR components resulted in decreased parasite replication and blocked effector export into the host cytosol. GRA45, the putative chaperone describe above, is needed for membrane association of MYR components (Wang et al. 2020). The other components, GRA44, PPM3C, and a rhoptry protein ROP17 could play a role in regulating translocation or effector activity through phosphorylation. ROP17, a serine/threonine protein kinase residing on the host cytosol side of the PVM, is speculated to phosphorylate one or multiple components of the translocon as ROP17 kinase activity is needed for its role in translocation (Panas et al. 2019). GRA44 contains a putative phosphatase domain that interacts directly with GRA45 (Coffey et al. 2018), however the targets of GRA44 phosphatase activity have not been identified (Blakely et al. 2020). PPM3C is also a phosphatase and in its absence, PPM3C target proteins GRA16 and GRA28 exhibited changes in phosphorylation, and were not exported into the host (Mayoral et al. 2020). While phosphorylation of MYR1 was also altered in the PPM3C knockout, a global defect in effector export was not observed as GRA24 and IST1 export was unaffected. PPM3C knockout parasites exhibited growth defects *in vitro* and reduced virulence in mice. It has yet to be determined which components of the MYR translocon are structural and which are regulatory. Cryo-EM studies, like those performed with the *Plasmodium* translocon (see below), would be extremely informative.

In *Plasmodium falciparum* the machinery for GRA protein export into the PV has been extensively investigated and there are a number of significant differences between *Toxoplasma* and *Plasmodium* translocons that are worth highlighting. The majority of *Plasmodium* proteins transported past the PVM have both a signal sequence and a N-terminal *Plasmodium* export element (PEXEL) found about 15–30 residues downstream of the signal sequence (PEXEL: RxLxE/Q/D or relaxed PEXEL: RxLxEE) (Boddey et al. 2016; Boddey and Cowman 2013; Boddey et al. 2010; Russo et al. 2010; Sleebs et al. 2014).

Only a very small number of exported proteins in *P. falciparum* parasites do not have a PEXEL motif. Most of these PEXEL-negative export proteins (PNEPs) also do not contain an ER-signal sequence but it has been shown that the first 20 amino acids at the N-terminus of PNEPs and one or more transmembrane domains are enough to promote export (Heiber et al. 2013; Boddey and Cowman 2013; Hiller et al. 2004). The aspartyl protease Plasmepsin V (PM5), which is a homolog of TgASP5 and localized in the ER, cleaves the PEXEL motif after the leucine residue, leaving the protein with an acetylated xE/Q/D N-terminal motif (Boddey and Cowman 2013; Boddey et al. 2010; Russo et al. 2010; Sleebs et al. 2014). PNEPs or proteins with newly exposed N-terminal PEXEL motif are targeted to *Plasmodium* translocon of exported proteins (PTEX) embedded in the PVM (Koning-Ward et al. 2009; Marti and Spielmann 2013). PTEX is composed of the 3 core proteins EXP2, PTEX150, HSP101, as well as 2 other proteins PTEX88 and TRX2 (Elsworth et al. 2014). EXP2 is a heptameric complex in which each monomer contains a single transmembrane domain (Ho et al. 2018). EXP2 and PTEX150 form an interdigitating protein conducting channel through which effector proteins are translocated through the PVM into the host (Ho et al. 2018). HSP101 is an AAA ATPase that binds and provides the energy needed for protein translocation and plays a role in unfolding proteins prior to translocation (Bullen et al. 2012; Chisholm et al. 2018; Elsworth et al. 2014; Ho et al. 2018; Sanders et al. 2019). Refolding of effector proteins happens in the host, although the mechanisms behind this process are not fully understood. Host proteins and exported parasite chaperones, such as HSP40 and HSP70, are thought to play a role (Hakamada et al. 2020; Külzer et al. 2012). GRA17 and GRA23 are related to *Plasmodium* EXP2. However, as described above these proteins do not play a role in protein translocation in *Toxoplasma*, but rather form a pore in the PVM to allow diffusion of small molecules (Gold et al. 2015). The function of two additional PTEX components, PTEX88 and TRX2, are less well defined. In *P. berghei*, PTEX88 and TRX2 are nonessential while in *P. falciparum* TRX2 knockouts exhibited a reduction in protein export (Chisholm et al. 2018; Elsworth et al. 2014; Matthews et al. 2013; Matz et al. 2013).

Phosphorylation of GRA Proteins in the PV

Proteomic analysis has demonstrated that a large number of GRA proteins are phosphorylated upon secretion, many of which are differentially phosphorylated in tachyzoites and bradyzoites (Treeck et al. 2011; Young et al. 2020). The effect of phosphorylation in regulating GRA function has only begun to be appreciated. A number of kinases and phosphatases are secreted from the dense granules, including GRA44 and PPM3C described above, and the newly characterized WNG1 and WNG2 kinases (Beraki et al. 2019). Originally thought to be rhoptry kinases targeted to the host cytosol (Peixoto et al. 2010), ROP34 and ROP35 have been reclassified as it has recently been demonstrated that they are secreted from the dense granules and localize to the PV lumen (Beraki et al. 2019; Coffey et al. 2018). Phosphoproteomic analysis identified a number of GRA proteins that exhibited differential phosphorylation patterns in WNG1 knockout parasites (previously ROP35) including GRA6. In the absence of WNG1, GRA6 was not inserted into the IVN and remained in the PV lumen which lead to defects in IVN development. WNG2 (previously ROP34) contributes to parasite virulence *in vivo*, however the targets of WNG2 have not been identified (Coffey et al. 2018).

Strand forming proteins within the PV have recently been described that are separate from the IVN. However the role it plays in the parasite life cycle is unclear (Young et al. 2020). These strands form through interactions between the C-termini of two dense granule proteins, strand-forming protein (SFP1), and GRA29 (Young et al. 2020). Phosphorylation negatively regulates the formation of the SFP1-GRA29 strands (Young et al. 2020). The enzymes responsible for this phosphorylation have not been identified.

Regulation of host cell cycle

T. gondii infection results in significant transcriptional changes in infected cells and dense granule proteins play pivotal roles in mediating this response.

Cell cycle control: A number of GRA proteins have been characterized that alter the signaling pathways controlling the host cell cycle. *T. gondii* infection leads to host cell cycle arrest in G2 and inhibiting the G1/S transition led to decreased parasite proliferation (Brunet et al. 2008). The combined action of two dense granule effectors (HCE1/TEERG and GRA16) controls G2 arrest. Identified through bioinformatic approaches, GRA16, localizes to the host nucleus after translocation from the PV where it interacts with host proteins PP2A and HAUSP (Bougdour et al. 2013). HAUSP (herpesvirus-associated ubiquitin-specific protease) binds to the transcription factor and tumor suppressor p53 (Li et al. 2002). In healthy cells, p53 levels are low due to continuous degradation by the proteasome. Upon infection or DNA damage HAUSP deubiquitinates p53 leading to p53 stabilization, nuclear accumulation and transcription of p53 responsive genes. HAUSP-GRA16 interactions leads to p53 stabilization which leads to decreased levels of Cyclin B, a factor which controls the G2/M transition of the cell cycle. (Bougdour et al. 2013). TEEGR/HCE1 is also trafficked to the nucleus, where it binds to the E2F-DP1 transcription factor (Braun et al. 2019; Panas et al. 2019) leading to increased cyclin E expression, which drives infected cells through the G1/S cell cycle transition. It is not understood why arresting cells in G2 confers a proliferation advantage to parasites and warrants further investigation.

PP2A, the second host protein that binds to GRA16, is a component of the holoenzyme that controls phosphorylation and stability of c-myc, a vital transcription factor that controls the expression of a wide variety of genes involved in cell cycle regulation, and apoptosis (Bougdour et al. 2013; Panas and Boothroyd 2020). *T. gondii* infection upregulates c-myc expression (Franco et al. 2014) but significantly reduced c-myc expression was observed in GRA16 knockout parasites. In addition, expression of TgGRA16 in *Neospora caninum* conferred this parasite with the ability to upregulate c-myc (Panas and Boothroyd 2020).

Regulation of immune response pathways

Parasite proteins that alter the host immune response can be broadly categorized into two groups: Those found at the PVM that limit PV destruction by both host Immunity-Related GTPases (IRGs) and Guanylate Binding Proteins (GBPs) (Guevara, et al. 2021); and those that are secreted into the host nucleus and cytosol to influence immune signaling and transcriptional pathways (Hakimi et al. 2017).

Modulation of IRG and GBP: The mediators of host IRG and GBP inactivation are predominately secreted from the rhoptries (Saeij et al. 2006; Taylor et al. 2006). The active kinase ROP18 is secreted into the host cell during invasion where it complexes with inactive pseudokinases ROP5 (Behnke et al. 2011; Reese et al. 2011) and ROP8/2 on the cytosolic side of the PV to phosphorylate IRGs and perturb their recruitment to PV. The demonstration that GRA7 binds to this rhoptry complex was one of the first examples of an interaction between rhoptry and dense granule proteins (Alaganan et al. 2014; Dunn et al. 2020). These proteins have distinctive and complementary roles in host immune evasion (Alaganan et al. 2014).

Manipulation of immune signaling pathways: Dense granule proteins manipulate key immune-related signaling pathways including the NF-kB, IFN-γ, and p38 MAPK. GRAs have opposing activity on the pro-inflammatory NF-kB pathway. In type II strains, GRA15 induces nuclear translocation of NF-kB resulting in upregulation of pro-inflammatory cytokines including IL-12 and IL-1β (Rosowski et al. 2011). A second dense granule protein, the previously mentioned TEEGR/HCE1, which binds E2F-DP1, represses the expression of a subset of NF-kB effectors (Braun et al. 2019; Panas et al. 2019). These opposing effects can be explained by the parasites need to limit cytokine activation and prevent tissue damage, after all the parasite transmission on the host depends on host survival and parasite encystation in tissues of the host.

Independent of the NF-kB signaling, parasites also control IL-12 production by manipulating the MAPK pathway (Braun et al. 2013). GRA24 localizes to the host nucleus upon secretion from the PV where it binds directly with the MAP kinase p38a where it facilitates p38 autophosphorylation in the absence of the typical kinase cascade that controls MAPK activation (Cuadrado and Nebreda 2010). This results in increased IL-12 production.

IFN-γ is another central player in immune signaling. Pathways induced by IFN-γ culminate in nuclear translocation of the transcription factor STAT1 and upregulation of STAT1 responsive genes. Upon *T. gondii* infection STAT1 dependent transcription is inhibited by the dense granule effector IST1 (Inhibitor of STAT1 Transcriptional activity). In the absence of IST1, *T. gondii* survival in activated macrophages is reduced (Olias et al. 2016). Export of IST1 from bradyzoites protects infected cells from IFN- γ mediated cell death (Seizova et al. 2022).

Antigen presentation: Antigen presentation is a vital immune-related process that leads to the destruction of infected cells. Infected cells present protein fragments on their surface via MHC-1 pathway for recognition by CD8+ T cells. PV localized dense granule and rhoptry proteins have the ability to suppress antigen recognition in host CD8+ T cells, while also being processed for presentation (Blanchard et al. 2008; Feliu et al. 2013; Frickel and Hunter 2021; Gregg et al. 2011; Grover et al. 2014; Rommereim et al. 2019). In the absence of ROP18, ROP5 and GRA7, the PV is targeted for host cell destruction by host IRG's leading to increased antigen presentation (Rommereim et al. 2019). PV associated proteins are also processed for presentation from intact PV's although it is not understood how proteins are released into the cytosol for degradation and entry into the MHC-1 pathway. However, GRA2, GRA3 and GRA12 play a role in mitigating presentation, as antigen

presentation is increased in knockout parasites, although the mechanisms underpinning this phenomenon are not understood (Rommereim et al. 2019; Lopez et al. 2015). In GRA2 knockout parasites, IVN formation is disrupted leading to increased GRA6 association to the PVM and increased GRA6 presentation via the MHC-1 pathway. Thus, the IVN may limit antigen presentation by modulating MHC-1 processing of PVM bound proteins(Lopez et al. 2015).

Bradyzoite cyst formation and maintenance

GRA protein function has been predominately investigated in the fast-replicating tachyzoite form of the parasite, which is responsible for the acute infection and vital for parasite dissemination into peripheral organs. In the presence of a functioning immune system tachyzoites differentiate into the slow-growing, cyst-forming bradyzoites which can have lifelong persistence within skeletal, heart, and brain tissue (Di-Cristina et al. 2008; Watts et al. 2015). GRA proteins play vital roles in cyst formation and maintenance. During differentiation, a cyst wall forms on the luminal side of the PVM. The cyst wall consists of a dense outer layer, and looser filamentous layer underneath. The cyst lumen (also referred to as the cyst matrix) contains filamentous strands, vesicles and tubule structures termed the intracyst network (ICN) that is equivalent to the tachyzoite IVN (Lemgruber et al. 2011; Tu et al. 2020).

During the tachyzoite-to-bradyzoite transition, many IVN associated GRA proteins including GRA1, GRA2, GRA4, GRA6, GRA9, and GRA12 relocalize from the IVN to the cyst wall early in differentiation (Guevara et al. 2019; Tu et al. 2020). GRA2 expression is necessary for GRA4 and GRA6 localization to the cyst periphery (Guevara et al. 2019) and deletions of all but GRA1 resulted in slower cyst maturation (Guevara et al. 2019; Guevara et al. 2021). The localization of SFP1 and GRA29 also exhibit distinct localizations in tachyzoites compared to bradyzoites. In bradyzoites, these proteins do not form strands and are dispersed throughout the PV (Young et al. 2020). MAG1, first characterized over 25 years ago, is present in the PV lumen of tachyzoites. Upon differentiation, MAG1 expression is upregulated and localizes to the cyst wall and matrix, where it is thought to have a structural role (Parmley et al. 1994; Tomita et al. 2021).

Recent proteomic, transcriptomic and interactome approaches have identified a plethora of bradyzoite specific GRA proteins (Buchholz et al. 2011; Nadipuram et al. 2020; Tu et al. 2020), including MCP3, CST2, CST3, CST4, CST6 (Tu et al. 2019), GRA55–59 (Nadipuram et al. 2020), and CST7–9 (Tu et al. 2020). MCP3 knockouts appear to form smaller cysts with no obvious fitness loss (Tu et al. 2020), CST2 knockouts have attenuated virulence and do not establish a measurable cyst burden in mice (Tu et al. 2019), and GRA55 knockouts have a lower cyst burden in mice (Nadipuram et al. 2020). Bradyzoite pseudokinase-1 (BPK1) associates with the cyst wall and interacts with other cyst components MAG1, MCP4, GRA8 and GRA9. BPK1 knockout parasites formed smaller cysts that were more susceptible to pepsin digestion, leading to reduced efficiency of oral infection (Buchholz et al. 2013).

While knockouts of some of the cyst wall components have a demonstrated role in cyst formation or maintenance, the exact roles these proteins play, be they regulatory

or structural, is not understood. In addition, many GRA proteins exhibit differential phosphorylation in the bradyzoite stage vs. tachyzoite stages (Guevara et al. 2019; Young et al. 2020). Whether phosphorylation regulates GRA function or influences the redistribution of GRA proteins from the IVN to the cyst, and within the cyst wall during cyst maturation requires further investigation.

The export of effector proteins from bradyzoite cysts into the host cell is not well understood. In parasites induced to differentiate in cell culture, MYR1 was present on the bradyzoite membrane at both early (1 day) and late (7 days) stages of differentiation, allowing for the export of effectors from cysts. GRA16, GRA24, GRA28, and IST1 accumulated in the host early after induction but only IST1 was present in the host at later time points (Mayoral et al. 2020). However, it's not clear if this change in effector accumulation was due to changes in the MYR complex activity or due to changes in effector expression (Mayoral et al. 2020). The export of IST1 throughout early and late stages of bradyzoite infection has recently been confirmed to be MYR-dependent, supporting a role of the MYR complex in specific effector export during the bradyzoite stage (Seizova et al. 2022). Contrasting this, evidence of MAG1 secretion in both bradyzoites and tachyzoites in a MYR independent manner has been observed. The mechanism behind this MYR independent secretion warrants further investigation (Tomita et al. 2021). In addition, GRA17 is found in the cyst membrane and postulated to provide a similar role in diffusion of solutes from the host as described in tachyzoites (Gold et al. 2015; Paredes-Santos et al. 2019).

While GRA proteins are secreted from bradyzoites, it is unclear if there are differences in DG biogenesis, granule transport dynamics, and regulation of secretion between tachyzoites and bradyzoites.

Parasite Egress/Calcium homeostasis

GRA1 was the first dense granule protein identified. It's found in the PV lumen upon release and contains a predicted EF-hand calcium binding domain (Cesbron-delauw et al. 1989). It's predicted to be essential for parasite growth in culture, however its function and putative role in calcium homeostasis have not been elucidated (Sidik et al. 2016).

Parasite egress from the PV is triggered by increased intracellular calcium and secretion of proteins from the micronemes. However, four GRA proteins have been implicated in egress (Bisio et al. 2019; LaFavers et al. 2017; Okada et al. 2013; Schultz and Carruthers 2018). GRA41 is associated with the IVN and GRA41 knockout parasites show defects in IVN formation and exhibit a dysregulation in take up of extracellular calcium (LaFavers et al. 2017). However, GRA41 is not thought to bind calcium directly so the dysregulation may be an indirect consequence of loss of GRA41. GRA22 knockout parasites also exhibit defects in the timing of egress (Okada et al. 2013). Interestingly, GRA22 also does not contain putative calcium binding domains, so the mechanism by which this protein controls egress is not understood. TgLCAT knockout parasites do not display proper egress phenotypes, with its catalytic activity being required (Schultz and Carruthers 2018). Lastly, Diacylglycerol Kinase 2 (DGK2) is secreted into the PV and produces phosphatidic acid, which acts as a signaling molecule for egress (Bisio et al. 2019).

Concluding Remarks:

Dense granules proteins are vital for regulating the intracellular environment of host cells. Less than a decade ago, only 25 GRA proteins had been identified (Mercier and Cesbron-Delauw 2015). Thanks to recently developed proteomic and transcriptomic approaches and CRISPR-Cas9 technology, that has made tagging and deletion of genes more efficient, the identification and characterization of GRA proteins has accelerated (Bai et al. 2018; Mayoral et al. 2021; Nadipuram et al. 2020; Naor et al. 2018; Shen et al. 2014; Sidik et al. 2014; Sidik et al. 2016). However, the function and mechanism of action of many GRA proteins is poorly understood, particularly in the bradyzoite stage of the parasite lifecycle, and there are undoubtedly more GRA proteins yet to be discovered.

A more complete understanding of dense granule biology will require a range of experimental approaches: additional proteomic analysis for further identification of GRA lumen and membrane associated proteins, live cell imaging to visualize DG secretion to directly measure rates of dense granule secretion from both tachyzoites and bradyzoites, biochemical and biophysical approaches to characterize GRA protein properties and protein-protein interactions, and in vivo studies to access the roles of GRA proteins in parasite dissemination in the host and cyst formation. Collectively, this will lead to a more complete understanding of *T. gondii* pathogenicity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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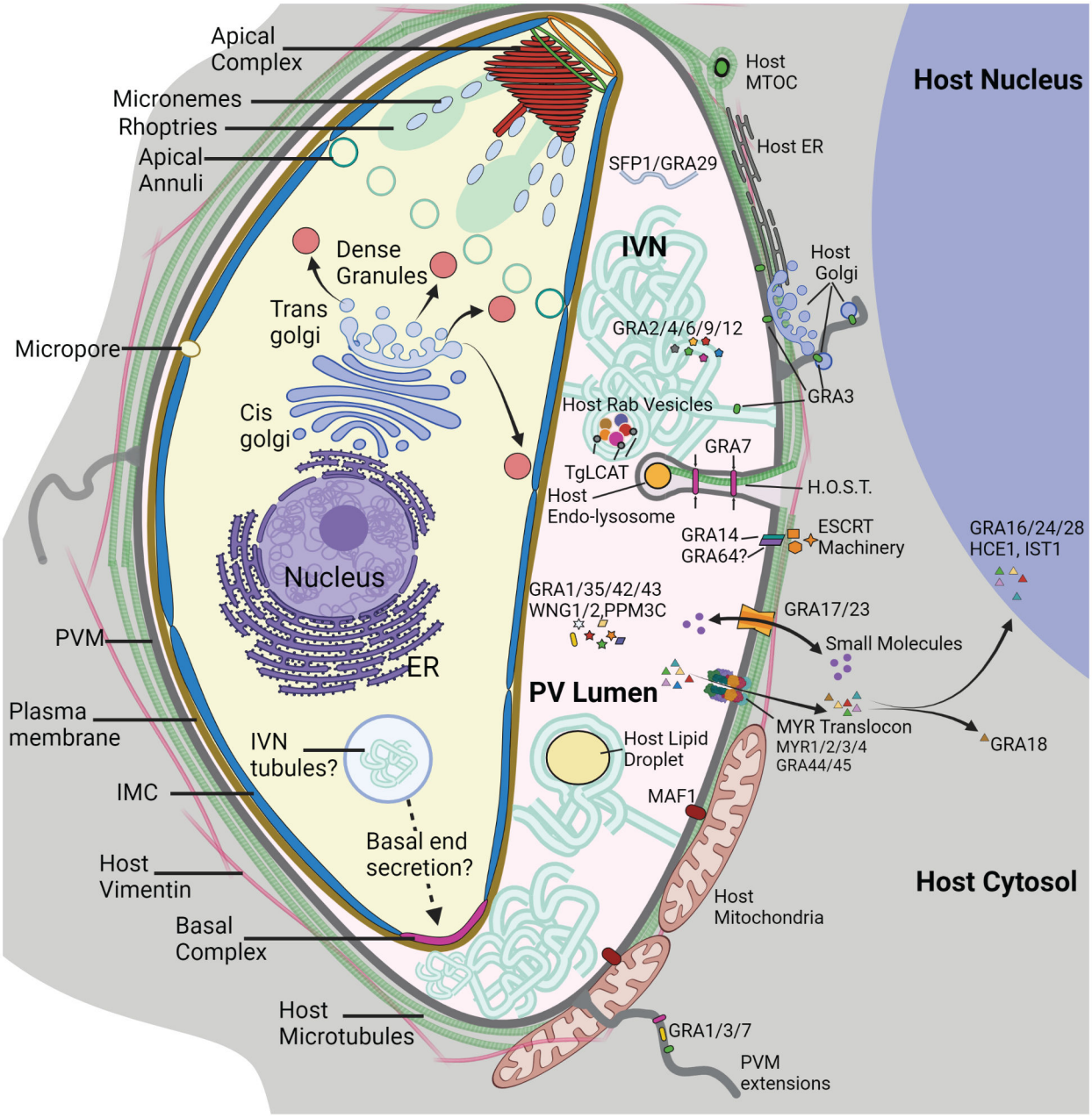

Figure 1.
Illustration of parasite (tachyzoite stage) and parasitophorous vacuole morphology

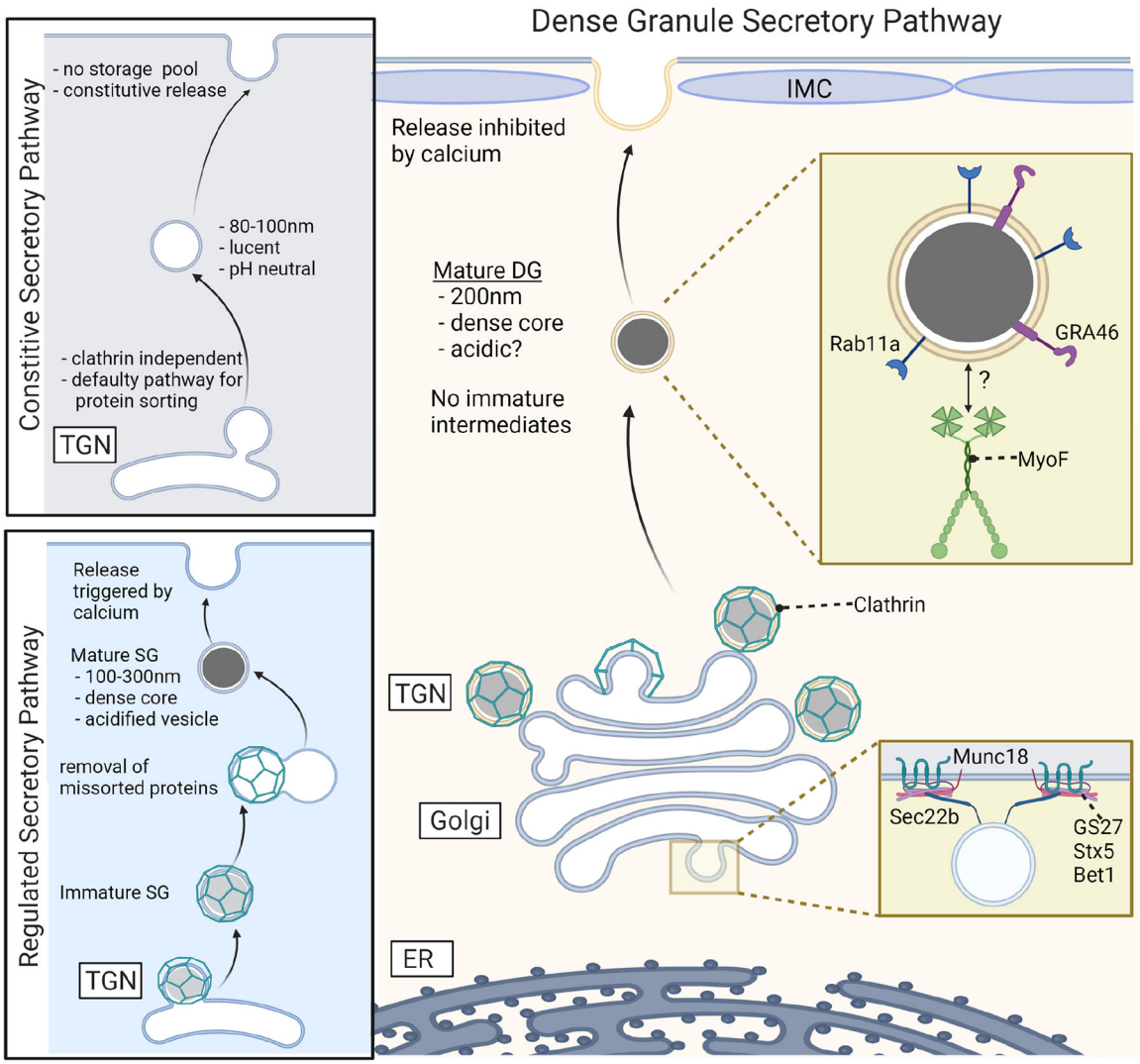

Figure 2.

Model of dense granule biogenesis and trafficking compared to constitutive and regulated secretory pathways in higher eukaryotes.