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## THESE

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VENUGOPAL Kannan

# Role of the Clathrin Adaptor Complex AP1 and the small GTPase Rab11A in Anterograde Trafficking in *Toxoplasma gondii*

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Composition du jury:

Madame le Docteur LEBRUN Maryse, Rapporteur

Monsieur le Docteur SILVIE Olivier, Rapporteur

Madame le Docteur SOLDATI-Favré Dominique, Examinateur

Monsieur le Docteur MEISSNER Markus, Examinateur

Monsieur le Docteur DELEVOYE Cedric, Examinateur

Madame le Docteur MARION Sabrina, Directeur de Thése

Laboratoire de Biologie Cellulaire et Moleculaire du Toxoplasme Centre d'Infection et d'Immunité de Lille, Institut Pasteur de Lille CNRS UMR 8204 – INSERM U1019

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## Résumé

Toxoplasma gondii, l'agent causal de la toxoplasmose appartient au phylum des Apicomplexes. Comme son nom l'indique, le parasite possède un complexe unique d'organites sécrétoires apicaux, les micronèmes, rhoptries et le conoïde, qui jouent un rôle essentiel pour l'invasion de la cellule hôte et la survie du parasite dans la vacuole parasitophore. T. gondii est devenu un modèle populaire de biologie cellulaire et aussi un outil de référence pour l'étude de l'organisation ultra-structurale et des différentes fonctions des autres parasites du phylum Apicomplexa tel que Plasmodium, l'agent causal de la malaria. Des progrès récents dans la dissection des différentes voies du trafic intracellulaire ont conduit à une meilleure compréhension de la biogenèse des organites de sécrétion. Cette thèse porte sur deux facteurs essentiels à la survie du parasite : le complexe adapteur de la clathrine AP1 et la petite GTPase Rab11A, qui jouent un rôle crucial dans la régulation de certaines voies du trafic intracellulaire. Ainsi, nos travaux ont permis de démontrer un rôle pour AP1 dans le triage différentiel et le transport vésiculaire des protéines MIC et ROP depuis le Trans-Golgi-Network (TGN) et les compartiments endosomaux. D'autre part, nos résultats ont révélé un rôle original de AP1 dans la division parasitaire aux stages tardifs de la cytokinèse. Nous avons également identifié un partenaire de AP1, la protéine unique de T. gondii possédant un domaine ENTH : EpsL (pour Espin-Like Protein). Dans les autres Eucaryotes, les protéines epsines sont connues pour activer la formation des vésicules à clathrine en co-opération avec les complexes AP1 et AP2. Nos résultats préliminaires ont suggéré un rôle de EpsL, similaire à AP1, pour assurer la formation des rhoptries et micronèmes. Nous avons, dans un deuxième temps, examiné les différentes fonctions de la petite GTPase Rab11A. Notre étude par vidéomicroscopie sur cellules vivantes, semble indiquer que Rab11A régule le transport de vésicules depuis le TGN vers la périphérie cellulaire et en particulier, les pôles basal et apical du parasite. Après sur-production de la forme mutée inactive de Rab11A, notre étude a révélé un nouveau rôle de la protéine dans la sécrétion des protéines membranaires de surface et dans l'exocytose des granules denses, lors de l'invasion de la cellule hôte mais aussi durant la réplication parasitaire. Finalement, des expériences de pull-down ont permis d'identifier un partenaire intéressant liant Rab11A seulement sous sa forme activée, la protéine unique de T. gondii contenant un domaine HOOK (TgHOOK), décrit pour lier les moteurs moléculaires de type dynéine. Nos résultats préliminaires suggèrent que TgHOOK pourrait réguler le transport des vésicules positives pour Rab11A d'une manière dépendante des microtubules. Par conséquent, cette étude a permis de révéler de nouveaux aspects, encore inexplorés, bien qu'essentiels, des mécanismes régulant la sécrétion de molécules à la surface parasitaire.

# **Summary**

Toxoplasma gondii, the causative agent for the disease Toxoplasmosis belongs to the phylum Apicomplexa. As the name implies, the parasite possesses a unique complex of apical secretory organelles namely the micronemes, rhoptries and conoid, which favor host cell invasion and intracellular survival. T. gondii has become a popular cell biology model and also a reference tool for studying the structure and functions of other important parasites that belong to the same phylum, such as plasmodium, but also higher eukaryotes. The recent advances in dissecting protein trafficking pathways have led to a better understanding of the biogenesis of apical organelles and also to the identification of crucial protein molecules that could determine the fate of the parasite. This thesis focuses on two different molecules, the Clathrin Adaptor complex AP1 and the small GTPase Rab11A that play a crucial role in distinct trafficking pathways of the parasite contributing to a wide range of functions. First, we demonstrated a role of AP1 in the differential sorting of microneme and rhoptry proteins at the Trans-Golgi-Network and endosomal level. Accordingly, depletion of AP1 leads to a defect in apical organelle biogenesis. In addition, we revealed an original role of AP1 in parasite division by regulating late stages of cytokinesis. We also identified and studied a partner of AP1, the unique ENTH domain containing protein of the parasite, EpsL (for Espinlike protein). In other Eukaryotes, epsin proteins are well known regulators of clathrinmediated vesicular budding in co-operation with AP1 and AP2. Our preliminary results suggested that EpsL shares similar functions to AP1 in the regulation of rhoptry and microneme formation. We next worked on the small GTPase Rab11A and defined the dynamics of the protein within the parasite by live imaging. In addition to its known role in cytokinesis, we unravelled a novel function for the molecule in the secretion of surface membrane proteins and the exocytosis of dense granules during both, parasite invasion and replication. Further, pull down experiments on active Rab11A helped us fish an interesting partner molecule, the unique HOOK-domain containing protein that we characterized for the first time in T. gondii. Our preliminary data suggest a role of Rab11A in microtubuledependent transport of vesicles in a HOOK-regulated manner. Therefore, our study provides novel molecular insights into a yet unexplored but essential aspect of constitutive secretion in the parasite.

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# **Scientific Output**

## **Poster Presentations**

- « Role of Rab11A and AP1 in replication and host cell invasion by *Toxoplasma gondii.* » \* First biannual conference of ParaFrap in Les Embiez islands, Marseille from the 29<sup>th</sup> September to 2<sup>nd</sup> October.
- « Role of the *Toxoplasma gondii* clathrin adaptor AP1 in the differential sorting
  of rhoptry and microneme proteins and in parasite growth. »
  - Poster presentation at the 27<sup>th</sup> Molecular Parasitology Meeting 2016, Woodshole, USA from the 18<sup>th</sup>- 22<sup>nd</sup> September 2016.
  - Poster presentation 2<sup>nd</sup> biannual conference of Labex ParaFrap in Les Embiez islands, Marseille from the 2<sup>nd</sup> 5<sup>th</sup> October 2016.
- « HOOKed to Rab11A dynamics and novel secretory functions of Rab11A in *Toxoplasma gondii* »
  - Turbo Talk and poster presentation at the 27<sup>th</sup> Molecular Parasitology Meeting 2016, Woodshole, USA from the 18<sup>th</sup>- 22<sup>nd</sup> September 2016.
  - Poster presentation at the 2<sup>nd</sup> biannual conference of Labex ParaFrap in Les Embiez islands, Marseille from the 2<sup>nd</sup> 5<sup>th</sup> October 2016.

## Articles

- Venugopal K, Tomavo S and Marion S. «Biogenesis of Secretory Apical Organelles in Toxoplasma gondii »: Toxoplasma gondii/host cell interactions: Recent advances. Editors: C. Mercier and MF Delauw. Bentham Science Publishers, eBooks Section. Book chapter Accepted for publication
- Dual role of the *Toxoplasma gondii* clathrin adaptor AP1 in the differential sorting of rhoptry and microneme proteins and in parasite division. Venugopal K.<sup>1</sup>, Werkmeister E.<sup>1</sup>, Barois N.<sup>1</sup>, Saliou J.M.<sup>1</sup>, Sindikubwabo F.<sup>2</sup>, Hakimi M.A.<sup>2</sup>, Langsley G.<sup>3</sup>, Lafont F.<sup>1</sup>, and Marion S<sup>1</sup>.

<sup>1</sup> Centre d'Infection et d'Immunité de Lille, Université de Lille, CNRS UMR 8204, Inserm U1019, CHU Lille, Institut Pasteur de Lille, F-59019 Lille, France

<sup>3</sup> Laboratoire de Biologie Cellulaire Comparative des Apicomplexes, Faculté de Médicine, Université Paris Descartes - Sorbonne Paris Cité, France. Inserm U1016, CNRS UMR8104, Cochin Institute, Paris, 75014 France.

The article has been submitted to PLOS Pathogens and is under revision.

<sup>&</sup>lt;sup>2</sup> IAB, Team Host-pathogen interactions & immunity to infection, Université Grenoble Alpes, INSERM U1209, CNRS UMR5309, 38700 Grenoble, France

# **List of Abbreviations**

AMA-1 Apical Membrane Antigen 1 **AP** clathrin Adaptor Protein complex **APR** Apical Polar Ring **ARF** ADP Ribosylation Factor **BSA** Bovine Serum Albumin **CBL** Chitin-Binding-Like protein **CDPK** Calcium-Dependent Protein Kinase CRISPR/Cas9 Clustered Regularly Interspersed Short Palindromic Repeats/ CRISPR associated protein 9 DAPI 4',6-Diamidino-2-Phenylindole **DD** Destabilization Domain **DHFR** Dihydrofolate Reductase-thymidylate synthase gene **EGF** Epidermal Growth Factor **ER** Endoplasmic Reticulum F-actin Filamentous actin FBS Fetal Bovine Serum **GAP** Gliding Associated Protein **GEF** Guanine nucleotide Exchange Factor **GFP** Green Fluorescent Protein **GPI** Glycosylphosphatidylinositol **GST** Gluthatione S-Transferase GT1 Glucose Transporter 1 HA Haemagglutinin **HEK** Human Embryonic Kidney **HFF** Human Foreskin Fibroblasts HOPS Homotypic fusion and vacuole protein sorting IFA Immunofluorescence Assay **IFN** Interferon **IMC** Inner Membrane Complex **IP** Immunoprecipitation

**IRG** Immunity Related Guanosine Triphosphatases LB Luria-Bertani MAPK Mitogen-Activated Protein Kinase MAR Microneme Adhesive Repeat **MHC** Major Histocompatibility Complex MIC Microneme protein MJ Moving Junction MLC1 Myosin Light Chain-1 MPA Mycophenolic Acid **MPP** Microneme Protein Protease **MT** Microtubule **MTOC** Microtubule Organising Center MyoA MyosinA NSF N-ethylmalimide Sensitive Factor **ORF** Open Reading Frame **PAF** Paraformaldehyde **PAGE** Polyacrylamide Gel Electrophoresis **PAN** Plasminogen Apple Nematode **PBS** Phosphate Buffered Saline PCR Polymerase Chain Reaction **PFA** Paraformaldehyde **PM** Plasma Membrane **PV** Parasitophorous Vacuole **PVM** Parasitophorous Vacuole Membrane **RBC** Red Blood Cell **ROM** Rhomboid-like protease **RON** Rhoptry Neck protein **ROP** Rhoptry bulb protein **RT-PCR** Reverse-Transcriptase PCR SAG Surface Antigen Glycoprotein **SDS** Sodium Dodecyl Sulphate **SEM** Scanning Electron Microscope **SIM** Structured Illumination Microscopy SNARE Soluble NSF Attachment protein Receptor

SUB Subtilisin-like protease TAE Tris/Acetate/EDTA TGN Trans Golgi Network TRAP Thrombospondin-related Anonymous Protein VPS Vacuolar Protein Sorting YFP Yellow Fluorescent Protein

# **Chapter I- Introduction**

## 1. The Apicomplexa

The apicomplexa constitute a large phylum of obligate intracellular protozoan parasites that cause several debilitating diseases in humans and livestock and claim millions of lives every year. The phylum derives its name from the unique complex of organelles that the parasites house at the cell apical region, namely the micronemes, the rhoptries, the conoid, and the apical polar ring (APR) which aid parasite invasion and survival in host cells (Figure1; Morrisette et al., 2003). The most popular among this phylum are *Plasmodium falciparum* the causative agent for human malaria, a devastating tropical disease caused by the bite of infected female anopheles mosquitoes, and *Toxoplasma gondii* the causative agent for toxoplasmosis, a predominantly opportunistic parasitic disease affecting immuno-compromised individuals such as those who are infected by HIV or undergoing chemotherapy.



**Figure 1.** Apicomplexan parasites. This panel of electron micrographs is adapted from the works of Scholtyseck and Aikawa and presented on the webpage of the Morrisette lab. It depicts *Toxoplasma* responsible for human toxoplasmosis, *Lankestrella* which infects amphibians and birds, *Eimeria* which infects cattle and poultry, *Isospora* known to infect canines, felids and humans, *Sarcocystis* a parasite of mammals, reptiles and birds, *Babesia* which predominantly infects cattle and *Plasmodium*, the causative agent for malaria. The typical morphological features of an apicomplexan parasite is also depicted with key organelles such as the apical polar ring, the conoid, the micronemes, the rhoptries and the evolutionarily retained chloroplast like organelle- the apicoplast.

*Cryptosporidium* is also an apicomplexan parasite which causes opportunistic infections in immunocompromised individuals and leads to waterborne diarrhea. Tick borne*Theileria* 

species *T. annulata* and *T. parva* cause among cattle tropical theileriosis and East coast fever respectively, which lead to a great economical burden for the livestock and agriculture-based industries. *Babesia* and *Eimeria* cause babesiosis and coccidiosis respectively in cattle and poultry among other animals. There are also unique species of *Babesia* and *Eimeria* that are capable of infecting and causing diseases in humans.

## 2. Toxoplasma gondii and toxoplasmosis

*Toxoplasma gondii* is an obligate intracellular protozoan parasite that causes the disease toxoplasmosis. The parasite was first identified by Charles Nicolle and Louis Manceaux at the Pasteur Institute in Tunis in 1908. They found the parasite in the tissues of a hamster-like organism called *Ctenodactylus gundi*. The same year the parasite was also identified by Splendore in Brazil, in the tissues of a rabbit. The word *Toxoplasma* is derived from the Greek word "toxon" which means "bow", referring to the crescent shape of the organism (Black and Boothroyd, 2000), and "plasm" meaning form and hence together "*Toxoplasma gondii*".

### 2.1 Classification

Taxonomically, the parasite is classified as follows:

**Domain** Eukarya - The group of organisms with a true nucleus and membrane bound organelles.

**Kingdom** Alveolata - The alveolates are characterised by a unique set of membranous sacs that lie beneath their plasma membrane. The closely packed sacs are called alveoli.

**Phylum** Apicomplexa - Owing to the special set of organelles located at the apical region of the parasite.

**Class** Coccidia - The members of this class are obligate intracellular parasites.

**Order** Eucoccidiorida - The species under this order all go through asexual (merogony), sexual (gametogony) and spore forming (sporogony) stages during the life cycle.

**Family** Sarcocystidae – The members of this family require more than one obligatory host to complete their full life cycle. The host participates in a predatory/prey relationship. Oocysts are passed between them through faeces.

Genus Toxoplasma

**Species** gondii

There are primarily three different clonal lineages of *T. gondii* that have been widely studied, namely Type I, Type II and Type III, and they differ from each other in their virulence and epidemiology (Howe D.K and Sibley D.L., 1995). Type I includes the most extensively characterized GT-1 and RH strains, Type II includes ME49 and its derivatives such as PDS and PLK, and Type III is represented by VEG and CEP (Saeij J.P.J. et al., 2005). These strains have been completely or partially sequenced and the genome database has been made available on <a href="http://www.toxodb.org">http://www.toxodb.org</a>.

### 2.2 T. gondii life cycle

The parasite life cycle is divided into the sexual and asexual phases that occur in the feline intestine and mammalian/avian populations, respectively. The sexual phase of the parasite life cycle is hosted by the members of the Felidae family, for example domestic cats. The male and female gametes fuse to produce the zygote or oocyst within the intestinal epithelial cells. The unsporulated oocysts when shed by the cat via its faeces reach the environment. Sporogony follows, when the unsporulated oocyst matures under certain environmental conditions to produce infectious oocysts, each of which contain two sporocysts, which in turn contain four sporozoites each (Jackson M.H. and Hutchinson W. M., 2000, Dubey J.P. and Frenkel J.K., 1972). While unsporulated oocysts are resistant to adverse environmental conditions, sporulated oocysts are sensitive (Dubey J.P. et al., 1970, Ito et al 1975). Ingestion of oocysts via contaminated water or food by any warm-blooded animal including livestock and humans leads to the asexual phase of the parasite life cycle. The asexual phase is characterised by two distinct stages of growth, which correspond to the acute and chronic states of infection. During the acute phase following the rupture of the ingested oocysts, the released sporozoites develop into the endozoites or tachyzoites, which represent the rapidly growing form of the parasite. The very special feature of the tachyzoite is its ability to invade nearly all type of cells and even fish and insect cell lines (Werk R. 1985) and to grow within a protective vacuole lined by the parasitophorous vacuolar membrane (PVM). The tachyzoite goes through multiple replication cycles in a process called endodyogeny wherein two daughter parasites bud from within the mother parasite (detailed in chapter 4.3.2) (Ogino and Yoneda, 1966). This unique division process repeats itself several times in a wide range of cell types, producing the heavy parasitemia that is responsible for the various clinical manifestations of the disease. If the parasite is challenged by stress conditions such as the host immune response, the tachyzoites differentiate into latent bradyzoites encapsulated in sturdy cysts. The cysts may remain dormant for the entire life span of the intermediate host or rupture and convert again to the tachyzoite forms to cause disease conditions, upon a lowered immune response as in immune-compromised humans (Blader I.J. et al, 2015). The cysts are mostly localized in muscular and neural tissues or the eye, but can be also detected in visceral organs such as lungs, liver and kidneys (Dubey J.P., 1993). In the horizontal transmission route, bradyzoite cysts contained in the intermediate hosts (for example, mice) can be consumed by either a definitive or an intermediate host by carnivorism. When bradyzoites are ingested by a definitive host, the asexual phase resumes as the parasite differentiates in accordance to its new host and the sexual life cycle is initiated.



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**Figure 2.** *Toxoxoplasma gondii* **life cycle (Hunter CA and Sibley DL 2012).** The definitive host for *Toxoplasma gondii* belongs to the Felidae family (most commonly the domestic cats) where the sexual phase of the parasite occurs. The merozoites replicate inside the enterocytes of the cat intestine in a process called merogony and as a part of the maturation process some differentiate into the male and female gametes, which eventually fuse to produce the diploid oocysts. The unsporulated oocysts shed in the cat's feces sporulate and undergo meiosis in non-physiological conditions resulting in haploid sporozoites. The oocysts, which are extremely durable in external environmental conditions can possibly be consumed by rodents or farm animals where the asexual phase of the parasite ensues, characterized by the fast growing tachyzoites, which are responsible for the acute inflammatory phase of the disease. The tachyzoites can also differentiate into the bradyzoite forms and remain dormant in the intermediate hosts. Man is infected by the parasite via contaminated water or food, which includes ingestion of infected farm animals by carnivorism.

### 2.3 Toxoplasmosis

#### 2.3.1 Modes of Transmission

T.gondii is transmitted to humans mostly via contaminated food and water. Infection occurs after the consumption of undercooked meat such as pork and lamb (beef and poultry animals have also been indicated in epidemiologic studies) that carries tissue cysts, or water and food contaminated with cat faeces containing ooycsts. Another mode of transmission is congenital where an infected mother transmits the parasite to the growing fetus via placental infection. The prevalence of congenital toxoplasmosis ranges from 1 to 10 per 10,000 livebirths (Guerina N.G. et al., 1994; Motoya J.G. and Liesenfeld O., 2004). The frequency of vertical transmission from a mother to the fetus is higher when the mother is infected during gestation or less than 3 months prior to gestation, however congenital toxoplasmosis is more severe when parasites are transmitted during the first trimester, having the potential to cause death of the developing foetus (Gavinet M.F. et al., 1997; Vogel N. et al., 1996). Thirdly, toxoplasmosis can be spread during blood or organ transplantations from sero-positive donors to sero-negative recipients (Motoya J.G. and Liesenfeld O., 2004). Due to immunosuppressive treatment, reactivation of latent infection is the cause for disease in patients with bone marrow, haematopoietic stem cell and liver transplants, or HIV infection. (Siegel S.E. et al., 1971). Occupational transmission via contaminated needles, labware or animal models has been reported as well (Kayhoe D.E. et al., 1957; Remington J.S. and Gentry L.O. 1970).

#### 2.3.2 Dissemination and Pathogenesis

During acute *T. gondii* infection, the ingested cysts or sporulated oocysts that reach the intestinal lumen, rupture to release the bradyzoites and sporozoites, respectively. The parasite then rapidly spreads from the initial site of infection, to the various near and far tissues by crossing different biological barriers such as the intestinal epithelium, the endothelial cells of the blood vessels, and organs such as the brain, eyes and placenta (Dubey J.P. et al., 1997; Harker K.S. et al., 2015). In an in vitro study, the tachyzoites have been reported to migrate across tight junctions of the intestinal epithelial cells by a paracellular route (Barragan A. et al., 2005). In addition, neutrophils harboring live non-replicating parasites were also shown to greatly enhance the spreading of parasites within the intestine and likely beyond (Coombes J.L. et al., 2013). Likewise, dendritic cells (DCs) harboring *T. gondii*, and particularly the Type II strain, were shown to exhibit elevated levels of migratory capacity and were proposed

to behave like 'Trojan horses' to facilitate parasite spreading, primarily to mesenteric lymph nods and the spleen (Lambert H. et al., 2006; Lambert H. et al., 2009). This observation and other studies added further understanding to the initial findings of lymphadenopathy in mice during acute T. gondii infection (Courret N et al., 2006; Zenner L. et al., 1998). Similarly macrophages infected with T. gondii also promote parasite dissemination to distal lymph nodes (Da Gama L.M. et al., 2004). Notably, tachyzoites stimulate macrophages and DCs to produce Interleukin 12 (IL-12), which activates Natural Killer cells (NK cells) and T cells to produce interferon gamma (IFN-y) (Gazzinelli R.T. et al., 1994) (Figure 3B). IFN-y together with tumor necrosis factor (TNF) trigger and activate macrophage and pro-inflammatory monocytes to mediate killing of tachyzoites (Sibley L.D. et al., 1991, Butcher and Denkers 2002) (Figure 3B.). In the bloodstream, tachyzoites were observed to preferentially infect monocytes than other leukocytes (Channon J.Y. et al., 2000; Sylveira C et al., 2011). The bloodstream flow helps the parasites carried by the monocytes to reach the vascular endothelium and further spread towards different organs, in particular the brain (Ueno N. et al 2014, Berenreiterova M et al., 2011). The parasite hosted by circulating monocytes is capable of crossing the blood brain barrier (Lachenmaeier S. et al., 2011). The local pro-inflammatory immune response, in particular IFN- $\gamma$  and TNF, which induce the production of Nitric oxide (NO), triggers the rapid conversion of tachyzoites into latent bradyzoites. Usually asymptomatic in immune-competent individuals, toxoplasmosis can lead to harmful effects on behavior and physiological responses. Reactivation of the brain cysts under immunesupressive conditions can lead to severe toxoplasmic encephalitis (Luft B.J. et al., 1992).

Retinochoroiditis (Butler N.J et al., 2012) is the most common clinical manifestation of *T. gondii* infection in adults or in new-borns during congenital toxoplasmosis (Ajzenberg D. et al., 2002). Human DCs infected with tachyzoites were found to migrate across the retinal endothelium by molecular interactions with ICAM-1(Intercellular Adhesion Molecule-1), VCAM-1(Vascular Cell Adhesion Molecule) and ALCAM (Activated Leukocyte Cell Adhesion Molecule) (Furtado J.M. et al., 2012).



**Figure 3A. Parasite infective stages across** *T. gondii* **life cycle (Gangneux, F.R. and Darde, M.L., 2012).** This illustration shows the three different infective stages of *T. gondii* across its life cycle. 1. Highlighted in pink - Self-limiting number of asexual replications (Schizogony) within intestinal epithelial cells of the definitive host is followed by sexual stage development (Gametogony). Following fertilization, the unsporulated oocysts are released into the environment via cat faeces. 2. In green -Sporogony occurs in environmental conditions, which includes a meiotic reduction and formation of sporulated oocysts. 3. In blue - Oocysts when consumed by intermediate hosts (rodents, mammals, birds) and following rupture, release sporozoites, which infect the intestinal epithelium and differentiate into tachyzoites. Tachyzoites spread, invade and replicate in any kind of cell asexually (Endodyogeny) and can convert into bradyzoites, which reside in cysts. These latent tissue cysts ingested by other intermediate or definitive hosts offer the continuum for the parasite life cycle.

In the case of congenital toxoplasmosis, all three strains of parasites, Types I, II and III were demonstrated to breach the placental barrier between the mother and the fetus via the small extravillous trophoblast layer (EVT) rather than via the large syncytiotrophoblast layer which is in direct contact with the mother's blood, suggesting that the parasite might enter the placenta via a constrained portal in the maternal-fetal interface (Robbins J.R. et al., 2012).



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**Figure 3B. Interferon**  $\gamma$  (IFN $\gamma$ ) mediated immunity to *T*, *gondii* infection (from Yarovinsky, F., 2014). Activation of macrophages and dendritic cells (DCs) after infection or by circulating antigens binding Toll Like Receptor 11 and 12 (TLR11 and 12), induces the secretion of IL-12. IL-12 triggers the massive production of IFN  $\gamma$  by different immune cells, such as the NK cells, the neutrophils, the CD4 and CD8 T cells and DCs. IFN $\gamma$  and the activation of CD8+ T cells are the essential parameters for resistance to *T. gondii* during chronic infections.

#### 2.3.3 Diagnosis and treatment

To diagnose *T. gondii* infection, several methods have been followed such as: serologic tests, PCR, histological examinations using immunoperoxidase technique, and to a lesser extent: antigen detection in body fluids, toxoplasma skin test, and antigen specific-lymphocyte transformation assay (Montoya J.G. 2002). Serological examination includes various techniques such as the Sabin Feldman Dye Test, Enzyme Linked Immuno Sorbent Assay (ELISA), immunosorbent agglutination assay (ISAGA), indirect haemagglutination assay (IHA), Western Blotting and IgG avidity (Liu Q. et al., 2015; Dard C. et al., 2016). As a part of a humoral immune response to *T. gondii* infection, the body produces immunoglobulins of different sub-classes: IgA, IgM, IgE and IgG, each presenting a specific kinetic profile following primary infection (Robert-Gangneux F. and Darde M.L. 2012). Although the above mentioned methods are not perfect and new methods are required, serologic tests based on detection of multiepitope chimeric peptides or antigens are the best for the moment (Dard, C. et al., 2016).

The most common treatment for toxoplasmosis is a chemotherapy, which involves administration of a combination of drugs including pyrimethamine and sulfadiazine plus folinic acid (Delden Van C. and Hirschel B., 1996). This treatment is mainly used for HIV positive individuals and also patients suffering from ocular and cerebral toxoplasmosis. Pregnant women within their first trimester are recommended spiramycin as this drug does not cross the placental barrier and pose teratogenic risks. Following the first trimester, either pyrimethamine or the triple drug treatment combining sulfadiazine, pyrimethamine, and folinic acid is administered (Rorman, E., et al, 2006). To patients who are hypersensitive to sulfadiazine, clindamycin is given as an alternative (Katlama C. et al., 1996). Macrolide and lincosamide based antibiotics and naphtoquinones can be also prescribed. Even though many of the available therapeutics are able to inhibit parasite replication, there is a constant need for drugs that can eradicate the parasite with a more specific mode of action (Antczak M. et al., 2016). New drug candidates such as pyrazolo pyrimidine (PP) and ATP competitive inhibitors, which function against the parasite essential enzyme Ca2+ Dependent Protein Kinase 1 (CDPK1) (Ojo K.K. et al., 2010), are on the rise. Plant derived compounds for long have been suggested as alternatives for synthetic drugs. Artemesia annua (Oliveira T.C. et al., 2009), Zingiber officinale (Choi K.M. et al., 2008), and Dictamnus desycarpus extracts are some of the plants that have shown to be highly effective against *T.gondii* infection (Antczak M. et al., 2016).

Until now there has been only one licensed vaccine against *T. gondii* for veterinary use called "TOXOVAX" which is a live attenuated vaccine based on a S48 strain (Buxton, D., 1993). When used in cats, it inhibits sexual development of the parasite. A challenging criterion for a potential vaccine candidate is that the antigen has to be available in all three major infectious stages namely the tachyzoites, bradyzoites and sporozoites. A recent study showed that using DNA vaccine boosted with recombinant adenovirus vaccine encoding ubiquitin conjugated antigens from the different infectious stages proved to be effective against type I and type II parasites (Yin, H. et al., 2015).

#### 2.3.4 Prophylaxis

To prevent congenital transmission, as a method of primary prevention, pregnant women need to be educated about sources of *T. gondii* infection and thus adopt respective prevention measures. This should include proper freezing and heating of meat based foods, washing of vegetables and fruits, hand hygiene following gardening or soil associated chores and precautions in cleaning cat litter boxes. This is indeed a list of general measures that should be

adopted by everyone. As a secondary method prenatal and postnatal serological testing has to be advised to pregnant women (Gajurel K. et al., 2015; Opsteegh M. et al., 2015). Cat owners should be careful about pet maintenance and make sure that they keep their cats indoors during the night and prevent them from consuming foods that carries the risk of being contaminated (Opsteegh M. et al., 2015). To protect HIV positive individuals, an antifolate drug combination including trimethoprim and sulfamethoxazole is administered. This combination is shown to prevent encephalitis as an outcome of reactivated *T. gondii* infection because of immune suppression.

### 3- Parasite Architecture and ultrastructural organisation

As previously mentioned, there are three different infectious forms that *T. gondii* takes during the course of its life cycle: i) the tachyzoites that divide rapidly and asexually, ii) the bradyzoites which form cysts that are capable of entering an indefinite period of latency and iii) the sporozoites which are contained in oocysts before rupture. The most widely studied tachyzoite form of the parasite is the subject of this thesis.

The term tachyzoite is derived from the Greek word "tachos" which means speed, signifying the rapid replicative stage where the parasite multiplies in continuous cycles. The parasite is  $\sim 8\mu m$  long and  $2\mu m$  in diameter (Joiner K.A. and Roos D.S., 2002).



Figure 4. The ultrastructure of *Toxoplasma gondii* tachyzoite. The scheme on the left (adapted from Baum J et al., 2006) shows the intracellular organization of the different organellar compartments and the cytoskeleton of a mature tachyzoite. The image on the right is an electron micrograph (adapted from Dubey et al., 1998) also showing the ultrastructure of the parasite contained in the parasitophorous vacuole (Pv), where the conoid (Co), micronemes (Mn), rhoptries (Rh), Golgi (Go), nucleus (Nu), nucleolus (No), dense granules (Dg), amylopectin granule (Am), lipid body (Lb) are visualized.

### 3.1 Intracellular organelles

With a haploid genome of ~63Mb packed in 14 chromosomes (Khan A et al., 2005 and Reid J.A et al., 2012), *T. gondii* possesses all the universal organelles of a typical eukaryotic cell such as a membrane bound nucleus, an endoplasmic reticulum that continues from the nuclear membrane, ribosomes, a single stack of Golgi network (Pelletier et al., 2002), and a mitochondrion (Melo E.J.L. et al., 2000) (Figure 4). However, the parasite also possesses special organelles such as the acidocalcisomes, involved in calcium homeostasis, cations and phosphorous storage, maintenance of intracellular pH homeostasis and osmotic regulation (Moreno, S.N.J. and Zhong, Li. 1996, Moreno, S.N.J. and Docampo, R., 2009); the plant-like vacuole (PLV), showing similar features as the digestive vacuole of yeast and plants. The PLV contains hydrolases, such as the Cathepisn L and plays a role in pH homeostasis and

calcium storage, particularly in extracellular parasites (Dou, Z. et al., 2014, Miranda, K. et al., 2010) and the apicoplast (Figure 4).

### **3.1.1** The apicoplast

The apicoplast is a secondary endosymbiotic organelle present as a relict plastid with four membranes among many members of the phylum apicomplexa including *T. gondii*. The *T. gondii* apicoplast encodes a 35Kb independent genome. However, several of the apicoplast specific proteins are in fact encoded by the nuclear genome. The metabolic pathways such as the FASII (Fatty Acid Synthesis), isoprenoid biosynthesis and haem are housed at the apicoplast. Owing to the evolutionarily conserved nature of these pathways of bacterial origin, the apicoplast has garnered attention as a favorite drug target over the years (Striepen B., 2011).

In addition, apicomplexans are characterized by a unique set of secretory organelles essential for parasite survival and virulence: the micronemes, the rhoptries and the dense granules.

### 3.1.2 Micronemes

Micronemes are small thread-like structures distributed along the cell cortex over the apical third of the parasite. Secreted microneme proteins (MICs) contribute to parasite adhesion to host cell surfaces, motility and host cell invasion (detailed in chapter 4.1.1). Proteomic analyses of gradient-purified organelle contents and excreted-secreted antigens have helped to identify and characterize the different MIC proteins (Bromley et al., 2003). The MICs carry out their different roles with the aid of different functional domains such as the microneme adhesive repeats domain (MAR), the thromspondin-1 type1 domain (TSR), which is present in glycoprotein adhesion molecules like MIC2 and MIC12, the von Willebrand A domain/ Integrin inserted domain A/I domain, Apple/PAN (Plasminogen, Apple, Nematode domain) (present in MIC4), EGF like domains (present in MIC3, 6, 7, 8, 9 and 12), and lectin domains such as the chitin binding like domain (CBL in MIC1, 3, and 8) (Garcia R.N. et al., 2000, Meissner M et al., 2002) (Figure 5).



Figure 5. Microneme (MIC) protein family (Carruthers V.B. and Tomley F.M., 2008). The image shows the architecture of the different known microneme proteins and their functional domains.

Microneme proteins generally function in complexes and ideally as a partnering of one transmembrane protein with one or more soluble proteins (Gaji R.Y. et al., 2011) (Figure 6). Further, using two-colored super-resolution Stimulated Emission Depleteion (STED) microscopy, it was recently shown that at least two sub-populations of micronemes can be detected in tachyzoites, containing different MIC proteins (Kramer, K. et al., 2013). Notably, the distinct distribution of MIC proteins might be directly correlated to spatio-temporal differences in their secretion during motility and invasion. Interactions between MIC proteins and host cell receptors are transient. Indeed, during parasite motility and invasion, the disassembly of the MIC protein complexes on the surface of the parasite from host cell receptors interaction has been shown to be mediated by cleavage via the activity of proteases. Primarily, the rhomboid proteases ROM4 and ROM5 found on the parasite plasma membrane, were shown to play a role in the proteolytic shedding process by cleavage of the transmembrane domain of MIC2 and MIC6 (Brossier, F. et al., 2005; Dowse, T.J. et al, 2005) (Figure 6) (see chapter 4.1.4).



**Figure 6. MIC protein complexes (Sheiner L et al., 2009)** The different microneme proteins that exist as complexes with partner molecules, and individual MICs such as MIC12 and MIC16 are depicted in this schematic representation. The regions where the different complexes are proteolytically cleaved is also depicted. The cleavage is mediated by proteases at a position within the luminal portion of transmembrane proteins such as MIC2, MIC6 and MIC12 and AMA1. The cleavage is accomplished during motility and the invasion process of the parasite. The well known candidates for this shedding process include rhomboid proteases TgROM4 and ROM5.

#### 3.1.3 Rhoptries

There are 8-12 rhoptries in each tachyzoite and each of them measure  $\sim 2-3\mu m$  in length (Dubey J.P. et al., 1998 and Boothroyd J.C. and Dubremetz J.F., 2008). They are club shaped organelles enclosed by a membrane with homogenous contents at the anterior portion, which is the neck region, and heterogenous contents at the base, which is the bulbuous region. The neck and bulb region proteins are thus respectively named as the RONs and the ROPs (Bradley et al., 2005, Dubremetz J.F., 2007). Of note, little is known about the mechanisms regulating de novo rhoptry biogenesis in replicating parasites (detailed in chapter 6.3). Notably, it has been recently shown that rhoptries are actively anchored at the apex of the parasite by the Armadillo Repeats Only Protein TgARO, in an actomyosin-dependent process (Mueller, C. et al., 2013; Mueller, C. et al., 2016). More than 30 rhoptry proteins have been

identified from subcellular fractionation studies (Leriche M.A. and Dubremetz J.F., 1991 and Bradley et al., 2005) and the list continues to expand. The rhoptry content also consists of membranous structures rich in cholesterol (Foussard et al., 1991). Rhoptry neck proteins participate in the formation of the moving junction (MJ), a transitory ring shaped adhesive structure that aids propelling the parasite into the host cell during invasion (detailed in chapter 4.2.1) (Alexander et al., 2005; Lebrun et al., 2005, Bargieri, D. et al., 2014). While the RON proteins favor invasion, the ROPs are secreted after the MJ is formed and targeted either, to the nascent parasitophorous vacuolar space, to the PVM, or to the cytoplasm of the host cell.

Most of the ROP proteins described so far are kinases or pseudo-kinases that serve many different functions to promote parasite intracellular survival by targeting host cell factors. There are also ROPs homologous to phosphatases (Protein phosphatase 2C PP2C) (Gilbert. L.A. et al., 2006) and proteases (Toxopain-1) (Xuchu, Q. et al., 2002). ROP1, ROP6 and ROP9 are exclusively unique to the Toxoplasma genus (Boothroyd and Dubremetz 2008). ROP6 was shown to be an intrinsically disordered protein without any characteristic functional domains (Lee, W.K. et al., 2014).

ROP proteins play an essential role in modulating host immune and metabolic responses upon invasion. ROP2 family proteins are an example for PVM targeted ROPs, involved in parasite protection against PVM degradation by the host cell. For example, ROP5, a pseudokinase, activates the kinase ROP18, which phosphorylates host IRG proteins (Immune related GTPases) maintaining in an inactive state thereby blocking PVM lysis (Fleckenstein, M.C. et al, 2012) (Figure 7). ROP18 also phosphorylates the ER stress response factor ATF6β, targeting it to degradation via the proteasome (Yamamoto, M. et al., 2011) (Figure 7). ROP16 and PP2C-hn (Protein phosphatase belonging to the 2C family; hn–host nucleus) are examples of the ROPs that reach the host cell nucleus. ROP16 was shown to interfere with the host STAT (signal transducer and activator of transcription) signaling pathway, thereby affecting the production of the key pro-inflammatory cytokine (IL-12) during *T. gondii* infection (Saeij et al., 2007; Gilbert et al., 2007) (Figure 7). The functional role of PP2C-hn has not yet been clearly elucidated. It was shown by knockout that the gene is dispensable for parasite virulence in vivo and that it is not essential to block host cell apoptosis (Gilbert, L. A. et al., 2007).



Figure 7 Rhoptry functions (Hunter, C.A. and Sibley, D.L., 2012). During invasion, secretion of the rhoptry neck proteins RON2, RON4, RON5 (and RON8, which was eventually identified) onto the host plasma membrane, together with microneme protein AMA1, triggers the moving junction formation. Rhoptry bulb proteins such as ROP5 and ROP18 are secreted into the parasitophorous vacuolar membrane. The PV resists acidification by fusion with host lysosomes or endosomes and also recruits other host organelles such as ER and mitochondria to scavenge nutrients. (Mordue, D.G. and Sibley, D.L. 1997). Type I strain ROP18<sub>1</sub> aided by ROP5<sub>1/III</sub> is on the cytoplasmic side of the PV and phosphorylates host IRGs (immunity related GTPases) preventing them from accumulating on the PVM and destroying the parasites. ROP18<sub>1</sub> also phosphorylates the host activating transcription factor ATF6β, which plays a role in the unfolded protein response (UPR) and might also serve an important role in regulating antigen presentation by dendritic cells. Other ROPs such as ROP16 JUII and PP2C-hn are targeted to the host cell nucleus. ROP16 J/III phophorylates STAT3 (signal transducer and activator of transcription3) and STAT6 resulting in prolonged activation of these transcription factors and eventually upregulating IL-4 (interleukin-4) and antagonising IL12. On the other hand, in type II parasite strains, the dense granule protein GRA15 activates TRAF6 (tumor necrosis factor receptor associated factor 6), which activates IkB kinases (IKK), causing phosphorylation and proteasomal degradation of IKB (inhibitor of NFKB). This releases NFKB (Nuclear Factor KB). NFKB translocates into the host nucleus and stimulates IL12 production.

#### **3.1.4 Dense Granules**

#### 3.1.4.1 Dense granule proteins localization and function

The dense granules are spherical electron-dense secretory vesicles distributed homogeneously throughout the parasite cytoplasm, which secrete their contents named as the GRA proteins. The dense granules are approximately 200 nm in diameter and are limited by a single membrane. According to the stage of infection, the number of dense granules present in a parasite may vary from approximately 15 in tachyzoites and sporozoites, 8-10 in bradyzoites and 3-6 in merozoites (Weiss L.M. and Kim K., 2011). Compared to micronemes and

rhoptries, the mechanisms regulating the biogenesis of dense granules remain unclear (detailed in chapter 6.6). On the other hand, similar to the ROPs, GRAs are also either targeted to the parasitophorous vacuole (PV), the parasitophorous vacuolar membrane and also beyond as far as to the host cell cytoplasm and nucleus. A recent review on the Dense Granule proteins by Corinne Mercier and Marie-France Cesbron-Delauw (2015) summarized the different sub-populations of the GRA proteins known so far and their functional roles. In particular, GRAs are essential to maintain the structure and integrity of the PV (Coppens I et al, 2006; Goldberg et al, 2015). They are found associated with the PVM (GRA3, GRA5, GRA8, GRA14, GRA19, GRA20, GRA21, GRA23), the H.O.S.T. (Host Organelle Sequestering Tubular structures) (GRA7) (Coppens, I. et al, 2006), or the membranous nanotubular intravacuolar network (IVN), which connects the parasites within the PV and to the PVM (GRA2, GRA4, GRA6, GRA9, GRA12, GRA23) (Mercier, C and Cesbron-Delauw, M.F. 2015; Masatani, T., et al., 2013). GRA1 is the only GRA protein present in a soluble form in the vacuolar space (Figure 8A and B). H.O.S.T. regulate the delivery of host endosomes and lysosomes to the PV. Disrupting the H.O.S.T. resulted in a loss of nutrient delivery from the host organelles and substantially reduced parasite growth (Coppens, I. et al,. 2006). Based on the observation that the nanotubular network is in contact with the limiting membrane of the PV, it was suggested that the IVN may participate in nutrient exchange between the parasite and the host cell (Sibley, D.L. et al., 1995), such as for lipid scavenging (Caffaro, C.E. and Boothroyd, J.C., 2011). The IVN might also play a role in maintaining the synchronicity of the parasite division events within the vacuole (Travier, L. et al., 2008). In addition, GRA proteins are essential to ensure parasite survival by modulating host cell functions. For example, GRA15 (Rosowski, E.E. et al., 2011), GRA16 (Bougdour A. et al., 2013) and GRA24 (Braun, L. et al., 2013) are secreted into the PVM and to the host cell nucleus, where they modulate the host immune response and cell division. While GRA15 influences the host NKKB pathway (Rosowski E.E. et al., 20111), GRA16 modulates cell cycle associated genes and genes associated with the p53 tumor suppressor pathway (Bougdour A. et al., 2013, Hakimi, M.A., 2015). On the other hand GRA24 mediates a sustained p38a MAP kinase activation pathway that modulates host immune response. Finally, GRA proteins also regulate host organelle recruitment at the PVM, such as the endoplasmic reticulum (Ahn, H.J. et al., 2006) and the Golgi apparatus, thereby promoting lipid scavenging from these organelles (Romano, J.D. et al., 2012; Julia, L. and Carruthers, V.B., 2008)



**Figure 8.** (A) (Adapted from Hammoudi, P.M. et al., 2015) This cartoon illustrates the different secreted dense granule proteins reaching the vacuolar space, the membranous nanotubular intravacuolar network (MNN or IVN), the PVM and the host cell nucleus. (B) The localization of the different identified GRAs is indicated in this immunofluorescence image showing a PV within an host cell (blue: host cell nucleus, green: parasite, white: parasite plasma membrane) (from Mercier C. and Cesbron-Delauw M-F., 2015)

#### 3.1.4.2 Dense granule proteins structure and secretion mechanisms

GRA proteins do not share high similarities in their sequence or conserved domains among themselves or with other proteins of known functions. However, Mercier, C. and Cesbron-Delauw, M.F. list out 16 GRAs (GRA1 GRA2 GRA3 GRA4 GRA5 GRA6 GRA7 GRA8 GRA9 GRA12 GRA14 GRA19 GRA20 GRA21 GRA23 and GRA25) as the 'canonical' GRAs, solely based on colocalisation with dense granules following IFA on intracellular and extracellular parasites. Apart from GRA20, the other GRAs possess either a classical or non classical N-terminal hydrophobic signal peptide which corresponds with their secretory nature, or other characteristic motifs which are shown in Fig 9. The other GRAs, which are targeted to the host cell nucleus, were considered as the non canonical GRAs (GRA15, It has GRA16 and GRA24). been shown that а ΗT (Host Targetting signal)/PEXEL(Plasmodium Export Element)-like motif present in GRA15 and GRA16 aids their export through the PVM (Coffey M.J. et al, 2015). In this extending list of GRA proteins, GRAs such as GRA11, GRA11bis and GRA12bis are yet to be characterized while others await identification (Mercier C. and Cesbron-Delauw M-F. et al., 2015).



**Figure 9. Dense Granule protein family** (Mercier C. and Cesbron-Delauw M-F. et al., 2015) Both the 'canonical' and 'non canonical' GRAs as identified in the Type II ME49 strains of parasites on ToxoDB are listed in this scheme with the different domains and motifs identified.

### 3.2 The T. gondii Cytoskeleton:

The cortical cytoskeleton of the parasite includes several components, which can be broadly categorized under two groups, 1) the pellicle, and 2) the sub-pellicular microtubule network with the apical and basal complexes.

### 3.2.1 The Pellicle

Classified as an Alveolate, the parasite possesses an inner membrane complex (IMC), a series of flattened membranous sacs located just beneath the plasma membrane (Ogino N et al., 1966). The plasma membrane together with the two membranes of the IMC constitutes the pellicle (Figure 9). The plasma membrane of the parasite is coated with diverse molecules that are anchored with a glycophosphatidyl inositol (GPI) moiety, such as the surface antigen-1 (SAG1) (Tomavo S. et al., 1989), SAG1-related sequences (SRS) (Manger I.D. Et al., 1998.), and SAG unrelated surface antigens (SUSA) (Pollard A.M. et al., 2008). These proteins

promote host cell adhesion and modulation of the early pro-inflammatory response in the intestine (Pollard A.M. et al., 2009; Rachinel, N. et al., 2004). Juxtaposed to the plasma membrane is the IMC, which serves as an anchor site for acto-myosin motors involved in parasite motility.



**Figure 10. The Pellicle.** The pellicle is composed of the Plasma Membrane (PM) and the Inner Membrane Complex (IMC) where is anchored the sub-pellicular microtubule network. (The electron micrograph is adapted from Johnson T.M. et al., 2007, and the graphic from Keeley A. and Soldati D., 2004).

The IMC is composed of three rows of longitudinal rectangular plates of flattened vesicles, which run down the parasite length with breaks at the apex and the base (Porchet E. and Torpier G. 1977; Del Carmen et al., 2009). The IMC is organised as a cone shaped apical cap, which is about 1µm in length, followed by 3 or 4 rows of six rectangular plates (Lentini G. et al., 2015). The plates are connected by transversal junction lines called sutures. Just below the plates are found 8-10nm filaments called alveolins (Gould et al., 2008), which form the subpellicular network (SPN) (Mann T. and Beckers C., 2001). The first alveolin to be localized at the SPN was TgIMC1 (Mann T. and Beckers C., 2001). Since then, by means of a systematic search, a total of 14 alveolin repeat containing proteins (TgIMC1 and TgIMC3-TgIMC15) have been identified (Anderson-White B.R. et al., 2010). The SPN is supposedly linked to the cytoplasmic face of the IMC plates (IMPs), which form a 32nm lattice also running the full length of the parasite. Adding to the list of proteins associated with the IMC, a family of three membrane tethered proteins called IMC sub-compartment proteins (ISP) were also identified (Beck J.R. et al., 2010).

#### **3.2.2** The microtubule network

Closely associated with the pellicle is a complex meshwork of microtubules and molecular structures at the apex and base of the parasite, which together preserve the structural integrity of the parasite and aid in motility and invasion of the host cells (Frixione E et al., 1996). The apical tip of the parasite holds a tube-like structure called the conoid, also a hallmark of the apicomplexan parasites. The conoid is topped by two preconoidal rings and has two ~ 400nm long microtubules passing through the middle and ending within the parasite cytoplasm (Morrissette, N.S. et al., 1997)(Figure 10B). TgCentrin2, an EF hand containing Ca2<sup>+</sup> binding protein, localizes at the anterior pre-conoidal ring, a series of annuli at the base of the apical cap of the IMC plates, and also at the basal complex. The protein is hypothesized to contribute to basal complex constriction during endodyogeny (Hu K., 2008). The membrane occupation and recognition nexus1 (MORN1) is localized at the apical end of the alveoli, spindle pole and also at the basal complex (Gubbels M.J. et al., 2006; Hu K., 2008). At the base of the conoid, the apical polar ring (APR) is present which serves as a microtubule organizing center, from where 22 microtubules emanate up to two-thirds of the body of the parasite, aligned in a counterclockwise direction following the conoid microtubule orientation in a minus end directed manner, taking into account the MTOC at the apex (Russell D.G., Buns R.G., 1984; Nichols and Chiappino, 1987).

#### 3.2.3. The conoid

The conoid by itself is a unique structure found in that is made up of 10 to 14 curved tubulin sheets (Hu, K. et al., 2002) and forms a hollow cone. The conoid, together with the preconoidal rings and the intra-conoidal microtubules forms a complex that can protrude beyond the APR or be withdrawn and surrounded by subpellicular microtubules (Morrissette, N., 2015). The protrusion event can be experimentally stimulated in extracellular parasites by Ca2<sup>+</sup> ionophores (Mondragon, R. and Frixione, E. 1996; Gonzalez DelCarmen et al., 2009). Interestingly, although present in *Toxoplasma* and other coccidians, the conoid is not present in *Plasmodium*, the closely related apicomplexan. Several proteins localise to the conoid, the intraconoidal microtubules and the preconoidal rings. Microtubules. In *Toxoplasma* both the minus end directed kinesin and plus end dynein motor proteins have been identified and particularly a dynein light chain 1 (DLC1 or DLC8a) was shown to be localized at the conoid (Qureshi, B.M. et al., 2013) (Figure 10 A). In general, dyneins are known to power axoneme

beating, organellar transport, spindle function and centrosome assembly (Hirokawa, N., 1998). ICMAP-1 is an intraconoidal microtubule associated protein, with which it was shown that the intraconoidal microtubules are formed during daughter parasite construction within the mother parsite. The functional characterisation of this protein is yet to be done. (Heaslip, A.T. et al., 2009). The intraconoidal microtubules might help in the release of secretory proteins by the anchorage of micronemes and rhoptries (Carruthers, V.B and Sibley, D.L., 1997;Nichols and Chiappino, 1987). SAS6L, SAS6 (centriole associated)-like protein, localizes to the preconoidal rings in tachyzoites (Leon, J.C. et al., 2013) and might play a role in anchoring SFA , a cell cycle regulated filament system which connects the conoid and the APR to the centriole in replicating parasites (Francia, M.E. et al., 2012).


**Figure 11.** *T. gondii* cytoskeleton (Anderson-White B. et al., 2012). The cytoskeletal structures distributed across the parasite are depicted in this image. **A**) The different subcompartments of the alveolar sacs are shown. The apical cap region with proteins occupying this region labeled in blue is distinguished from the three bands of alveolar sacs that follow below and also labeled with the respective markers. **B**) The structure of the conoid and the subpellicular microtubules is shown and all key protein markers of the structures labeled. **C**) The intramembrane particles (IMPs) lying within the alveolar vesicles, as well as the posterior cup containing the protein TgCentrin2, are shown. **D**) The distribution of the various IMC proteins and the TgCentrin2 annuli are depicted. BIC: Basal Inner Complex and BIR: Basal Inner Ring.

# 4. The Lytic Cycle

The lytic cycle of the tachyzoite includes a complex series of sequential events, which includes parasite adhesion and motility, host cell invasion, formation of the parasitophorous vacuole, parasite replication within the PV and finally egress from the infected cell. Each event is accomplished by the activities of a unique armada of parasite molecules.



**Figure 12. The Lytic Cycle of** *T.gondii* (Adapted from Billker O. et al., 2009). Tachyzoites employ gliding motility to move on cell surfaces and then invade. Adhesion is facilitated by microneme protein secretion. Invasion is achieved by the formation of a moving junction (depicted here as a tight constriction labeled in red) that propels the parasite into the host cytoplasm. The invasion process is contiguous with the formation of a parasitophorous vacuole within which the parasite undergoes active replication. Accumulation of abscisic acid (ABA) within the vacuole triggers parasite egress. Plant herbicide fluoridone (FLU) can inhibit ABA production and hence block egress, leading to development of semi-dormant cysts.

## 4.1 Gliding motility and adhesion

*T. gondii* employs a form of motility called the gliding motility which allows the parasites to move at a speed of ~  $1\mu$ m/s across cell surfaces (Hakansson et al., 1999). Gliding motility involves a series of events such as i) microneme secretion of adhesion molecules from the apical end of the parasite and insertion into the parasite plasma membrane, ii) translocation of adhesion molecules via the acto-myosin motors from apex to base, and iii) release of microneme proteins at the basal end after proteolytic cleavage by the rhomboid proteases (Blader I.J. et al., 2015; Meissner M. et al., 2013; Sharma P. and Chitnis C.E., 2013).

#### 4.1.1 Microneme secretion and parasite adhesion

The initial weak attachment of the parasite onto the host cell is mediated by GPI anchored surface antigen glycoproteins (SAGs), which bind to host sulfated proteoglycan receptors (He X.L. et al., 2002). Nearly 160 members of the SAG related sequences (SRS) have been identified, but the most widely studied has been SAG1 so far (Crawford, J. et al., 2009). More firm attachment is mediated by the microneme proteins (MICs), which bind to yet unkown carbohydrate receptors on host cell surfaces (Formaux et al., 1996; Barragan et al., 2005) (Figure 2.i). Transmembrane adhesin MIC2 has been the most widely studied for its role in parasite adhesion and motility in collaboration with its soluble partner M2AP (Huynh M.H. and Carruthers V.B. 2006). The secretion of micronemes is dependent on intracellular Ca2<sup>+</sup> levels (Wetzel D.M. et al., 2004). More precisely, following the parasite's initial contact with the host cell, extracellular potassium levels (Endo, T. et al., 1987) and other stimuli, trigger a signalling pathway activating phosphoinositide-phospholipase C (PI-PLC), which in turn generates second messengers such as diacyl glycerol (DAG) and Inositol 1,4,5 trisphosphate (IP<sub>3</sub>) (Lovett, J.L., et al, 2002; Bullen, H.E. et al., 2016). This causes a rise in the intracellular calcium level, which stimulates in turn the secretion of micronemes and also the protrusion of the conoid. Toxoplasma has its intracellular calcium stores at the ER, which is regulated by SERCA (sarco endoplasma reticulum Ca2<sup>+</sup> ATPase) (Nagamune, K. et al., 2007), and also in the acidocalcisomes (Moreno, S.N. and Zhong, L., 1996). Studies have demonstrated that when intracellular reserves of  $Ca2^+$  from the endoplasmic reticulum (ER) are depleted with thapsigargin, Ca2<sup>+</sup> influx from the exterior milieu can complement the intracellular levels and promote the essential events of microneme secretion, conoid protrusion, gliding motility, and host cell invasion (Pace, D.A. et al., 2014) (Moreno, S.N. and Zhong, L., 1996). Adding to the details of the calcium signaling pathway that is so critical towards essential parasite functions,

it was found that Ca2<sup>+</sup> activates the calcium dependent protein kinases (CDPKs), an apicomplexa specific kinase family, by binding to their EF hand domains. Conditional knock out of CDPK1 impaired calcium dependent secretion of the micronemes, which led to a blockade of parasite motility, host cell invasion and egress (Lourido, S., et al., 2010).

Following secretion, microneme proteins such as MIC2, which forms a complex with M2AP, have to be cleaved from their binding to host-cell receptors. Rhomboid potease 4 (ROM4) in particular was shown to be required for the cleavage of MIC2 transmembrane domain (Figure 6 and 13.1). ROM4 deletion mutants showed an accumulation of MIC2 on the surface of extracellular parasites and led to enhanced attachment, impaired motility (predominantly twirling) and impaired invasion (Shen B. et al., 2014; Rugarabamu, G. et al., 2015). However, the authors also suggested that MIC2 might still be cleaved by alternative ways which are yet unknown (Shen B. et al., 2014). In a similar manner, another protease TgSUB1(Subtilisin1) was shown to be required for the proteolytic processing of MIC2, MIC4 and M2AP. SUB1 deletion led to defective adhesion, motility and invasion.

## 4.1.2 The Glideosome: MyoA and MyoC glideosome complexes

Three different types of gliding motility have been described for the parasite on two dimensional surfaces, namely, circular gliding (full circular movements), helical gliding (half circular followed by a flip) and twirling (the parasite attaches to the cell surface with its base and spins its apex) (Hakkanson et al., 1999). On the other hand a different form of motility where the parasite moves in spiral cork-screw like trajectories was also observed in extracellular 3D gel matrix (Leung J.M. et al., 2014). The gliding motility is accomplished by a special molecular complex known as the glideosome. The glideosome is made of several components such as, the class XIVa myosin A, (Meissner M. et al., 2002) which is the key driving force, its regulatory light chain MLC1 (Myosin Light Chain1)(Herm-Gotz A et al., 2002), two essential light chains ELC1 and ELC2 (Williams M.J. et al., 2015) and the Glideosome Associated Proteins (GAPs): GAP40, GAP45, GAP50 (Frenal K. et al, 2011), and GAPMs (Bullen H.E. et al., 2009) (Figure 12). While GAP50 is anchored at the IMC, and GAP40 is a polytopic IMC resident, GAP45 is anchored at the plasma membrane (PM) and IMC via its N and C terminal extremities, respectively (Frenal K. et al., 2010) (Figure 13ii). The C terminal of GAP45 recruits MyoA and light chains MLC1 and ELC1 and ELC2 (Figure 12.ii). GAPMs are also localized at the IMC. Phosphorylation of MLC1 and Ca2<sup>+</sup>

binding by ELCs induce conformational changes and regulate MyoA activity (Nebl T. et al., 2011, Tang Q. et al., 2014). However, it was recently shown that the glideosome exists in three forms, distributed along the apical cap, central IMC and the basal pole. While the apical cap (which includes GAP70) and central IMC shared components of the MyoA glideosome, the basal pole had the MyoC glideosome (includes GAP80) (Frenal K. et al., 2014). It was also demonstrated that the Myo C and A glideosomes were able to compensate for the loss of each others' components showing the plasticity that aids parasite survival. The myosin component of the glideosome machinery pulls back on actin filaments ensuring the forward propulsion. In summary, for the myosins aiding motility or invasion, out of the eleven identified *T. gondii* myosin heavy chains (Foth B.J., 2005), MyoA (Meissner,M. et al. 2002), Myo C (Frenal K. et al., 2014) and MyoH (Graindorge, A. et al 2016) have been well characterized. Other myosins such as MyoB/C favor parasite replication (Delbac, F. et al., 2001), and Myo F is shown to function in centrosome positioning, apicoplast inheritance and dense granule intracellular trafficking (Jacot, D. et al., 2013; Heaslip, A.T., et al., 2016).

#### 4.1.3 Actin dynamics

Actin is predominantly in a globular form (Dobrowolski J.M. et al., 1997) distributed across the conoid, and as short dynamic filaments in the glideosome anchored at the IMC (Yasuda T. et al., 1988). *T. gondii* genome encodes only one actin gene, Act1, which has been described to exist predominantly in the globular form (G-Actin) rather than the filamentous form (F-Actin) (Dobrowolski J.M. et al., 1997). The polymerization-depolymerisation cycles of actin is very rapid in the parasite resulting in the formation of very short (100nm) unstable filaments (Sahoo N. et al., 2006). Thus F-Actin is predominantly seen in regions where the parasite is in contact with the host cell surface (Skillman K.M. et al., 2013). This correlates with the fact that the parasite lacks proteins such as the F-actin nucleation factor Arp2/3 that is responsible for actin branched network formation in other cell systems (Gordon J.L. and Sibley D.L., 2005). It was believed for a long time that the microneme protein complexes that favored adhesion and motility, interacted with actin via the glycolytic enzyme aldolase, which was however recently proven otherwise because of the dispensability of aldolase for host cell invasion (Shen B. and Sibley D.L., 2014) and hence leaving an open question.



## Figure 13. Activation of Gliding Motility and Invasion (Blader I.J. et al., 2015)

i) The attachment of the parasite to the host cell surface via SAGs and MIC protein, as well as, the cleavage of MICs by ROMs to promote parasite motility, are shown. ii) The organisation of the MyoA containing glideosome is depicted in this cartoon. The adhesion complex represents the MIC2-M2AP complex binding to the host cell receptor. The composition of the moving junction and its association with host cytoskeletal components, which participate to parasite invasion, is also shown.

Actin

Microtubules

### 4.2 Invasion

The parasite invades the host cell in 15 -30 seconds. Invasion of the parasite is powered by actomyosin motors and the contribution of micronemes and rhoptries in forming the moving junction, a transient structure that anchors the parasite to the host cell plasma membrane. Briefly, following the initial parasite attachment to host cell surface, the invasion process starts with the secretion of Rhoptry Neck proteins (RONs) into the host cell membrane, which interact with the secreted microneme protein AMA1 present on the parasite plasma membrane to form the moving junction. Following moving junction formation, the acto-myosin cytoskeleton activity aids the parasite in propelling itself forward. The rhoptry bulb contents (ROPs) are next injected into host cell and the parasitophorous vacuole encapsulates the parasite.



Figure 14. The invasion process (Carruthers and Boothroyd, 2007). This figure depicts the sequence of all the key steps involved in the invasion process of T.gondii in a time dependent fashion. All the events demonstrated in this figure from Step (3) are discussed below in detail.

## 4.2.1 Formation of the Moving Junction

AMA1 is anchored in the parasite plasma membrane and interacts with the RON complex anchored into the host cell plasma membrane following secretion from the rhoptry neck. The RON complex includes RON2, RON4, RON5 and RON8, where except RON2, anchored at the host plasma membrane, all the others are soluble (Straub K.W. et al, 2008). Structural studies have confirmed the direct interaction of RON2 with AMA1 and mapped the region wherein a conserved RON2 loop inserts deep into the hydrophobic groove of AMA1 (Tonkin M.L. et al., 2011). More recently, it was shown that there is also an AMA1 DII loop which acts as a structural gatekeeper to filter out other ligands that might bind with high affinity in the apical AMA1 groove (Parker, M.L. and Boulanger, M.J, 2015). Little is known about the host interactors of the RON 4,5,8 proteins in the host cytosol that allow the RON complex to be anchored at the host cortex. It was shown that RON4 interacts with the host cell  $\beta$ -tubulin and favors the invasion process (Takemae H. et al., 2013). Furthermore, in the absence of other MJ components, RON8, ectopically expressed in mammalian cells, trafficked to the cortical cytoskeleton at the periphery of the cell (Straub K.W. et al, 2008). It has to be noted that the moving junction composition continues to be a hot topic of debate. It is composed of the microneme protein Apical Membrane Antigen-1 (AMA1) and the rhoptry neck proteins (RONs). Recently there was a contradiction to this model wherein, by using a knock-out line, AMA1 was shown to be dispensable for MJ formation. The authors found that in absence of AMA1, all the RONs normally localized to the MJ with successful invasion (Bargieri D.Y. et al., 2013) suggesting the involvement of another MIC adhesin. Indeed Maryse Lebrun and coworkers demonstrated that the AMA1 knockdown was compensated by another AMA1 protein homologue, which also pairs with a novel RON2 homologue to ensure a functional moving junction and hence the successful invasion events (Lamarque M.H et al., 2014) (Figure 15). Overall, the moving junction is a well-conserved complex between the two major apicomplexans Toxoplasma and Plasmodium, with the key components such as, RON2, RON4, RON5 and AMA1 expressed by both the genomes (Basteiro, S. et al., 2011).



**Figure 15. Moving Junction (Lamarque, M.H. et al., 2014).** This illustration shows (a) a tahyzoite invading the host cell with the details of (b) the moving junction with the components involved in the formation. There are four AMA-RON interactions depicted here that span the parasite host interface, with the cytoplasmic tail of AMA1 connecting the complex to the parasite gliding machinery localized in the IMC. The RONs (RON4. RON5 and RON8 tethered to RON2) are beneath the host plasma membrane where they could interact with host cytoskeleton. While the complex AMA1-RON2 represents the primary pair used by Toxoplasma tachyzoites and Plasmodium merozoites, the other pairs are homologues. AMA3-RON2<sub>L2 and</sub> AMA4-RON2<sub>L1</sub> are highly expressed in sporozoite stage parasites. AMA2-RON2 and AMA4-RON2 <sub>L1</sub> have been demonstrated to compensate for AMA1 knock-out, showing the molecular plasticity of the MJ. "X" and "Y" represent yet to be identified components that can add to the complexity of the MJ.

#### **4.2.2 PVM formation**

The invasion process is correlated with the simultaneous formation of the parasitophorous vacuole within which the parasite will initiate its division, protected from the host immune response. An elegant study employing time resolved capacitance measurements and video-microscopy, demonstrated that the PVM consists of primarily invaginated host cell plasma membrane and pinching off of the PVM concludes the invasion process (Suss-Toby E et al., 1996). The moving junction aids the parasite to selectively filter out majority of the host cell transmembranous plasma membrane proteins and proteins associated with lipid rafts (Mordue D.G. et al., 1999), retaining only the lipids of raft and non-raft origin (Charron A.J. and Sibley D.L., 2004). This mechanism in principle provides a vacuolar compartment with a non-fusogenic membrane, free of receptors that could possibly interact with the host cell trafficking machinery. Indeed, the parasite was shown to import host cell endosomes and lysosomes via the formation of membrane invaginations of the PVM (H.O.S.T.) pushed into the vacuolar space by host microtubules, eventually fragmented by a dense collar of parasite proteins including GRA7 (Coppens, I. et al., 2006). This system allows protection from direct fusion and release of host hydrolases within the vacuolar space.

## 4.3 Intracellular parasite replication

Upon entry into the host cell, the parasite encapsulated in a parasitophorous vacuole starts to divide by endodyogeny, where the two daughter cells emerge within the mother cell without rupture of the nuclear membrane (Figure 16 A).

#### 4.3.1 A modified Cell cycle

With a haploid genome, the parasite undergoes a modified cell cycle in comparison to higher eukaryotes. The cycle includes G1, S and mitotic (M) phases but a true G2 phase between the S and M phases is lacking. Cyclins and cyclin-dependent kinases (CDKs) which are essential eukaryotic cell cycle regulators, have been identified in *T. gondii*, but which pairs of molecules control which step of the cell cycle has not been established (Kvaal C.A. et al., 2002; Blader I.J., 2015). It was discovered that the parasite possesses two distinct sub-transcriptomes which govern the cell cycle progression. The G1-subtranscriptome is for genes responsible for biosynthetic and metabolic functions and the S/M-subtranscriptome to meet functions such as DNA replication, de novo biogenesis of the intracellular organelles and

parasite egress. However it was also pointed out that one third of the genes are activated throughout the cell cycle and governed by the ApiAP2 transcription factors (Behnke M.S. et al., 2010). Cell cycle transcription in *T. gondii* happens in two waves, the first where mRNAs encoding genes involved in DNA replication, transcription and translation show maximum expression in G1 phase, whereas in the second wave the unique apicomplexan genes involved in constructing invasion (micronemes and rhoptries) and internal daughter structures, peak in the S/M phase (Behnke M.S. et al., 2010).

## 4.3.2 Centrosome initiated division process

The centrosome of the parasite is located closely apposed to the nuclear envelope where the spindle pole is embedded, and near the Golgi and apicoplast during interphase (Nishi M. et al., 2008). At the end of G1 the centrosome migrates to the basal pole of the nucleus divides and returns to the apical region where the spindle microtubules had been assembled and established. The existence of the centrosome in two distinct core complexes facilitates segregation of karyokinesis and cytokinesis and hence cell cycle flexibility. The outer core consists of TgCentin1-TgSfi1 pair, TgSas-6 and a novel Aurora-related kinase and the inner core is made of distant orthologs of CEP250/C-Nap protein family. The spatial relationship between the outer and inner cores is maintained throughout the cell cycle. As parasites pass into S phase, the cores duplicate sequentially starting with the outer core and making sure that each daughter receives one copy of each core. Further, a MAPK family Ser/Thr kinase restricts the core duplication to only once per cell cycle (Suvorova, E.S. et al, 2015). During this process, the Golgi and apicoplast elongation commence. At this point the daughter cytoskeletal elements begin to assemble over the duplicated centrosomes. Centrosome duplication and functional roles are regulated by several kinases such as the NIMA related kinase (Chen C.T. and Gubbels M.J., 2013), Aurora kinases (Berry L et al., 2016), Ca2<sup>+</sup> dependant kinase CDPK7 (Morlon-Guyot J. et al., 2014) and Mitogen activated protein kinase (MAPK-L1) (Suvorova E.S. et al., 2015). IMC15 (Anderson-White B.R. et al., 2010) and Rab11B have been reported to be the first markers to reach the outer centrosome, where Rab11B in particular has been demonstrated to transport vesicles from the Golgi to the developing IMC of daughter parasites (Agop-Nersesian C. et al., 2010). At the same time, the striated fiber assemblin (SFA), which forms the SFA fiber, anchors the centrosome at the base of the conoid of the budding cytoskeleton of daughter parasites (Francia M.E. et al., 2012). MORN1 a component of the centrocone, distributes to the apical and basal poles of the daughters (Gubbels M.J. et al., 2006; Hu K. 2008) (Figure 14 b). It plays an important role in daughter bud elongation and parasite segregation by regulating the assembly of the basal complex (Heaslip., A et al., 2010, Lorestani, A. et al., 2010), as well as apicoplast division (Lorestani, A. et al., 2010). Simultaneously ISPs are recruited to the daughter buds and particularly ISP2 was shown to be essential for IMC development (Beck J.R. et al., 2010). The inner membrane complex (IMC) is formed in two distinct steps -1. de novo assembly during the daughter IMC elongation within the mother cell; 2. Maternal IMC membrane recycling after the daughters have emerged from the mother cell (Ouologuem, D. and Roos, D.S 2014). The machinery involved in IMC biogenesis and recycling is still not completely clear. Two Rab11 isoforms Rab11A and Rab11B were shown to contribute to glideosome assembly and hence IMC biogenesis. Expression of Rab11A<sub>DN</sub> and Rab11B<sub>DN</sub> perturbed vesicular trafficking and cytokinesis (Agop-Nersesian, C. et al., 2009; Agop-Nersesian, C. et al., 2010) but the functional mechanisms behind these phenotypes is yet to be clearly dissected. As this process of daughter scaffold assembly progresses, a subset of IMC components transit to the basal complex, together with Centrin2. The basal complex and in particular the proteins MORN1 and Centrin 2, eventually tapers and constricts the basal pole of the newly formed daughter cells (Hu K., 2008). The basal complex does not fully separate the daughters, as a cytoplasmic bridge remains as the residual body. At the juncture where two daughter parasites have almost fully developed, the mother cell cytoskeleton begins to disassemble and the plasma membrane collapses between the daughter cells.

Simultaneously, the various organelles are duplicated and divided into the emerging daughter parasites with the aid of the new developing daughter cell scaffolds (Figure 16 C). This process takes place in a sequential fashion starting with the Golgi and apicoplast, then followed by the nucleus and ER, the mitochondrion and lastly the rhoptries and micronemes which are synthesized de novo and trafficked to the apex (Nishi M. et al., 2008) (see also chapter 6.2 and 6.3).



B





**Figure 16. Parasite replication A)** *T. gondii* endodyogeny process (Jacot D. et al., 2013). Endodyogeny begins with centrosome (Ct) duplication and Golgi fission. At the same time the apicoplast elongates with the extreme ends associated with the divided centrosomes. Nuclear division follows and the daughter cells emerge. Consistent with the constriction of the nucleus, the apicoplast divides into two. Rhoptries and micronemes are made *de novo* as a final step. The mother parasite's residual components are lost in the residual body. **B) Centrosome initiated division.** The centrosome coordinates both mitosis and cytokinesis simultaneously. The chromosomes are clustered at the centromeres and anchored to the spindle pole (centrocone) by the kinetochore. The organisation of daughter parasites is driven by assembly of the cortical cytoskeleton from apex to base. The nucleus is anchored in the daughter buds to taper towards the basal end and split the daughter parasites. The interaction between nucleus, centrosome, and daughter cytoskeleton is magnified. **C) Organelle duplication and division** was monitored using specific fluorescent markers and live cell imaging. The scheme in the lower panel summarizes the kinetics of Organelle duplication and division throughout the entire cell cycle (6-8 hours).

## 4.4 Egress

Several factors have been found to be responsible for triggering the egress of parasites. Firstly, host immune responses such as or perforin-dependent pathways, or death receptor (Fas/FasL) mediated damages to host cell triggered by the CD8<sup>+</sup> T response, can trigger parasite egress (Persson E.K. et al., 2007). Apart from a host immune response, other factors can influence egress after the parasite passing through several rounds of replication within a given parasitophorous vacuole. Indeed, it was shown that, as a result of continuous parasite secretion, a certain threshold level of abscisic acid (ABA) within the parasitophorous vacuole is reached and triggers parasite egress (Nagamune K. et al., 2008). Furthermore, a decrease in pH levels and hence acidification (Roiko M.S. et al., 2014) was shown to promote membrane binding and cytolytic activity of a perforin-like protein (PLP-1) secreted by the micronemes, which perforates host membrane and causes egress (Kafsack B.F. et al., 2009). Also NTPases (Nucleotide Triphosphate-degrading enzymes) secreted by parasites into the PV were shown to cause host cell ATP depletion and a Ca2<sup>+</sup> flux, inducing parasite egress (Stommel E.W. et al., 1997). Overall, several studies have proved that elevated intracellular Ca2<sup>+</sup> levels induce egress, promote motility and microneme secretion and also invasion capacity (Wetzel D.M. et al., 2004). More importantly the calcium dependent protein kinases (CDPKs) have garnered a lot interest since the last decade owing to their unique prevalence among apicomplexa and absence in the mammalian host, making them possible drug targets. TgCDPK1 and CDPK3 were shown to be essential for rapid activation of egress after induction by the Ca2<sup>+</sup> ionophore (Garrison E. et al., 2012; Lourido S. et al., 2012; McCoy J.M. et al., 2012).

# 5. Eukaryotic protein trafficking through the secretory and endolysosomal systems

Protein trafficking in eukaryotic cells is a highly organized process that is orchestrated across an endomembrane system, which includes secretory and endosomal-lysosomal components. The primary role of the secretory pathway is to enzymatically modify the cargo molecules at the Golgi and transport them across the cytoplasm to respective target destinations, which include intracellular organelles, the plasma membrane, and the extracellular space.



**Figure 17.** Protein trafficking routes across the anterograde and retrograde pathways (Ligeti, E. et al., 2012). In the classical anterograde trafficking route, the proteins synthesized from the ER are processed and transported via vesicles that bud from the Golgi network and delivered to the plasma membrane. Here, melanosomes, which ressemble lysosomal compartments are also depicted. In the endocytic pathway, cargos from the plasma membrane are routed either to the TGN or to the early endosomes via the sorting endosomes; in one of three possible ways: either clathrin dependent or independent internalization or mediated by caveolin. From the early endosomes, cargos that reach the recycling endosomes are returned back to the plasma membrane. Some cargos are targeted to degradation via the late endosomes and lysosomes. This transport occurs via the multivesicular bodies (MVB). Of note, particles internalized by phagocytosis are typically targeted to degradation, as it is also the case for the autophagosomes.

On the other hand, the endo-lysosomal system regulates the internalization of extracellular molecules and plasma membrane components, and their sorting for degradation to the lysosomes or for recycling to the plasma membrane via the recycling endosomal compartments or the TGN. (Burd, C.G., 2011). The Golgi apparatus is a central hub for protein maturation, processing and sorting events. It is made up of three layers namely the cis (early), medial (middle) and the trans (late) Golgi (Pfeffer, S.R., 2010). Particularly, the trans-Golgi-network (TGN) acts as an intersection between the secretory and the endo-lysosomal systems (Burd, C.G., 2011).

## 5.1 The anterograde trafficking pathway

The secretory trafficking mechanisms and molecules involved in the anterograde pathway are well conserved across different eukaryotic systems ranging from lower eukaryotes such as yeast to higher eukaryotes such as mammalian cells (Gadila, S.K.G. and Kim, K., 2016), with the exception of the plant system which present certain specific features. The primary variation includes a modified TGN which acts as an early endosome (Dettmer, J. et al., 2006) unlike the typical tubulovesicular early endosomes in mammalian cells (Reyes, F.C. et al., 2011). Of note, the TGN results from a Golgi maturation process of the trans-most cisternae that is ultimately released from the Golgi stack as an individual organelle (Kang, B.H. et al., Secretory proteins such as plasma membrane or lysosome targeted transmembrane 2011). proteins are synthesized in the rough endoplasmic reticulum (ER) and transported to the cis face of Golgi via a ER-Golgi intermediate compartment (ERGIC) in a coat protein II complex (COPII) dependent manner (Johnson, A. et al., 2015). It has also been proposed that COPI coated transport carriers (TCs) deliver cargo from ERGIC to the cis Golgi and also participate to their retrograde transport from the ERGIC to the Golgi (Brandizzi, F. and Barlowe, C. 2013; Zanetti, G. et al., 2012). On the other hand, for intra Golgi transport, cisternal maturation is the widely accepted model wherein Golgi cisternae are produced de novo and subsequently mature into the Middle and Trans Golgi Network (TGN) (Day, K.J. et al., 2013) with the Golgi resident cargoes being recycled from older to younger cisternae (Papanikou, E and Glick, B.S., 2014). Subsequent steps in the secretory pathway take place from the TGN. A classic example for cargo transport from the TGN to the early endosomes is the transport of the mannose 6 phosphate (M6P) receptor loaded with its cargos: lysosomal hydrolases containing M6P. The vesicle carrying the cargo is coated with clathrin and its adaptor protein (AP1) and targeted to the early endosomes (Gadila, S.K.G. and Kim, K., 2016). The M6P receptor is then recycled back to the TGN from the endosomes in a retromer and sorting nexin dependent manner (Seaman, M.N.J. et al., 1997; Arighi C.N. et al., 2004). In a similar manner, cargo molecules such as granzyme A, granzyme B, perforin and prosaposin are associated with sortilin, a lysosomal sorting receptor localized at the TGN that transports these molecules to the late endosomes and the lysosomes (Herda, S. et al., 2012;Braulke, T. and Bonifacino, J.S., 2009).

#### 5.1.1 Vesicle Budding at the TGN

Assembly of clathrin coated vesicles (CCV) at the TGN begins with the recruitment of Golgi resident small GTPase ADP ribosylation factor-1 (Arf-1) to the membrane. Arf-1 in turn recruits phosphatidyl inositol-4-kinase1 (PI4K), which is responsible for the production of phosphatidyl inositol-4-phosphate (PI4P). Functionally active Arf-1 and PI4P then recruit Golgi-localized y adaptin ear-containing ARF binding protein 2 (GGA2). GGA2 is responsible for recruiting more PI4K and hence increasing the local level of PI4P. Once PI4P level is optimally increased, AP1 is recruited (Daboussi, L. et al., 2012). GGA1 on the other hand has been shown to bind with Arf-3 (Boman. A.L. et al, 2000). AP1 associates with cargo-loaded receptors, based on two signature motifs such as the  $YXX\Phi$  and dileucine based [D/E[XXXL]L/I] which are present on the cytoplasmic tail of the receptor destined for endo/lysosomal compartments (Guo, Y. et al., 2014). The M6PR contains a tyrosine based motif crucial for the delivery of synthetic acid hydrolases from the TGN to the early and late endosomes (Ghosh, P. et al., 2003; Pandey, K. 2015). Next, clathrin coat proteins assemble to form a lattice meshwork that encapsulate the transport carriers (TCs) and trigger the formation of clathrin coated pits. An individual clathrin monomer is a triskelion made up of three heavy chains and three light chains. The triskelia polymerise to form polyhedral coats that can bud out of the TGN and transport cargo with all associated molecules to respective targets. Clathrin activates the membrane budding event at the plasma membrane and endosomes in association with the adaptor complexes AP1 and AP2 (detailed in chapter 5.2 and 5.3) (Royle, S.J. 2013). Epsins are part of the accessory molecules that activate the formation of clathrincoated vesicles together with the AP complexes. These proteins bind to specific phospholipids via their ENTH domain (Ford, M.G.J et al., 2002) and induce local membrane curvature (detailed in chapter 5.3).



Figure 18. Vesicle budding at the TGN (Gadila et al., 2016). A) Arf1 recruitment activates clathrin coated assembly at the TGN. Arf1 by itself also recruits PIK1 leading to low level of PI4P production. B) Arf1 and PI4P recruit GGA1 which interact with cargo receptors and the coat protein clathrin. C) The elevation of PI4P production stimulates recruitment of additional GGA1. AP1 recruitment also depends on Arf1 and PI4P. AP1 binds to cargo receptors carrying the [DE]XXXL[LI] motif. Clathrin coat formation is followed by recruitment of Dynamin2, which causes pinching of clathrin coated vesicles. D) Clathrin coated vesicles travel to endosomes.

## 5.1.2 Membrane tubulation, scission and transport

Actin cytoskeleton polymerization at the vicinity of the TGN offers the force needed to cause tubulation of membrane towards the cytoplasm (Anitei, M., et al 2010). Assembly of the actin cytoskeleton is mediated by the Arp2/3 in its phosphorylated form. Activation of Arp2/3 is induced by the WAVE/SCAR complex, a member of the Wiskott-Aldrich syndrome (WASP) family of proteins (Choi, C.H. et al 2013; Anitei, M., et al 2010). The precursor tubulation on the other hand is directed by the myosin protein Myo1b (Coudrier, E. and Almeida, C.G., 2011), which is targeted to the TGN via its pleckstrin homology (PH) domain, which recognized and bind the phosphoinositol PI(4)P (Greenberg, M.J and Ostap, E.M, 2013). The Myo1b head moves towards the plus end on the F-actin cytoskeleton and generates the force required for the tubulation event (Coudrier, E. and Almeida, C.G., 2011). Once the membrane curvature is accomplished via clathrin coat formation and actin polymerization, the other components further required for the tubulation process such as, the endophilin N-terminal amphipathic helix Bin/amphyphysin/Rvs (BAR) domain containing protein Arfaptin-1

(Gehart H et al., 2012) and the microtubule associated motor protein kinesin, are drawn to the membrane. Of note, the kinesin KIF13A was found to be associated with the  $\gamma$  subunit of the AP1 complex at the TGN to promote tubule elongation (Delevoye, C. et al., 2009) (Anitei, M. and Hoflack, B. 2011). It was hypothesized that the kinesin recruitment to the tubule tips in a microtubule-dependent manner, succeeds the previous event of actin-dependent Myo1b recruitment regulating nascent tubule formation (Almeida, C.G., 2011). Finally, the pinching or scission process of the CCV is accomplished by a dynamin. Dynamins constitute a superfamily of classical dynamins and dynamin-like or dynamin-related proteins (DLPs or DRPs) across Eukaryotes. The  $\alpha$  helix domain, one of the five domains of the classical dynamin protein, aids in forming helical oligomers (Gao, S. et al., 2010) by self assembly along the neck of invaginating or elongating membrane tubules (Mettlen, M. et al., 2009). Next, the GTPase activity of the GTPase causes the membrane constriction and pinching (Chappie, J.S. et al., 2010; Chappie, J.S. and Dyda, F. 2013). Finally, the TCs derived from the TGN are driven to the cell periphery in a microtubule dependent pathway.



**Figure 19. Membrane tubulation, scission (Gadila et al., 2016)** A) Membrane curvature at the TGN caused by clathrin coat assembly and accessory carriers, B) Clathrin and actin polymerisation generates the force necessary for the membrane deformation. The bar domain containing protein Arfaptin1 binds to the membrane and is responsible for the Arp2/3 mediated actin polymerization. C) Myo1b, a plus end directed motor, adds force to elongate the tubular membrane D) Next, Kinesin13A, another molecular motor binding to microtubules, exerts the final pulling force leading to tubule extension and scission with the help of Dynamin-2.

Different kinesins have been implicated in transporting TCs to the different target organelles. Kinesin-3 family member KIF13A was shown to be responsible for carrying the TCs containing M6PR and AP1 from the TGN to plasma membrane (Nakagawa, T. et al., 2000).

## 5.1.3 Vesicular Tethering, Docking and Fusion

The TCs or vesicles pinched off from the TGN and transported to the target organelles such as the endosomes or lysosomes have to next go through the processes of tethering, docking and fusion with the membrane of the target organelle. The tethering of vesicles to the membrane of the early endosome is mediated by the class C core vacuole/endosome tethering (CORVET) complex, a hexamer made up of six vacuolar protein sorting (VPS) proteins VPS11, VPS16, VPS18, VPS33, VPS3 and VPS8 (Figure 20). The first four of these six proteins, which are the Class C proteins are shared by the homotypic fusion and vacuole protein sorting complex (HOPS) (Perini, E.D. et al, 2014). The CORVET complex associates with SNARE proteins located on the vesicles at the TGN on one end and on the other end, via VPS3 and VPS8, with Rab5 on the targeted early endosomes (Bladerhaar, H.J. et al., 2012; Rana, M. et al., 2015). The CORVET complex was also shown to be involved in the homotypic fusion process between early endosomes (Kummel, D. and Ungermann, C., 2014). The tethering process is further aided by the Rab effectors Rabenosyn 5 and early endosomal antigen-1 (EEA-1). The tethering factors together with the SNAREs are essential for the docking of the Golgi derived vesicle with the early endosomes (Hong, W. and Lev, S., 2014). SNAREs play a vital role in the fusion process as well. There are around 38 SNAREs in the mammalian system, each displaying a distinct localization in different secretory and endocytic organelles. They are classified as v-(vesicular) and t- (target organelles) SNAREs or according to the amino acid content as R- (Arginine) and Q-(Glutamine) SNAREs (Malsam, J. and Sollner, T.N. 2011). The v- or A- SNAREs are known to interact with the t- or Q-SNAREs to form the SNAREpin or trans-SNARE complex in a zipper fashion (Shin, J. et al., 2014). The SNAREs thus mediate the fusion of the Golgi derived vesicles with the early endosomes. Another heterohexameric complex, the HOPS (homotypic fusion and protein sorting), shares four subunits with CORVET namely, Vps11, Vps18, Vps16 and Vps33, and has two other subunits Vps39 and Vps41. The HOPS complex is involved in binding vesicles to late endosomes and lysosomes via Rab7 (Balderhaar, H and Ungermann, C, 2013).



**Figure 20. Vesicular tethering, docking and fusion (Gadila et al., 2016)** A) Golgi derived vesicles are tethered to the early endosomes with the aid of the CORVET complex and Rab effectors. Tethering is initiated by Rab5 activation which is accomplished by the GEF activity of Rabaptin5-Rabex-5. Activated Rab in turn recruits the CORVET complex and the Rab effector EEA1 (early endosomal antigen1). The CORVET complex associates with SNAREs on the Golgi derived vesicle and it interacts with Rab5 on the early endosome. B) The CORVET complex makes use of its SNARE chaperoning activity to capture SNAREs and forms heterotetrameric trans-SNARE complex C) Once the trans-SNARE complex is formed, the Golgi derived vesicle fuses with the early endosome and empties its contents. D) The inset shows the different subunits of the CORVET complex.

#### 5,2 The Adaptor Protein Complexes

The adaptor protein (AP) complexes are heterotetrameric complexes, which play an important role in secretory and endocytic pathways. As described above for AP1, AP complexes recognize sorting signals in the cytoplasmic tail of cargo and in partnership with other accessory molecules, activate the vesicular transport of cargo from the donor compartment to different target organelle membranes. AP complexes connect cargo proteins and lipids to clathrin at vesicle budding sites, as well as binding accessory proteins that regulate coat assembly and disassembly (such as AP180, epsins and auxilin). So far, 5 different AP complexes have been identified and named as AP-1, AP-2, AP-3, AP-4 and AP-5. Except AP-4 and AP-5, the rest are clathrin-associated (Park, S.Y. and Guo, X., 2014). Each of the five AP complexes are made up of two large subunits – one each of  $\gamma$ ,  $\alpha$ ,  $\delta$ ,  $\varepsilon$  and  $\zeta$ , and  $\beta$ 1- $\beta$ 5, respectively, one medium sized subunit  $\mu$ 1- $\mu$ 5, and one small sized subunit  $\sigma$ 1- $\sigma$ 5. It was also found that some of these subunits have isoforms coded by different genes. AP-1 has two isoforms of  $\gamma$  ( $\gamma$ 1 and  $\gamma$ 2), two of  $\mu$  ( $\mu$ 1A and  $\mu$ 1B), and three of  $\sigma$  ( $\sigma$ 1A,  $\sigma$ 1B

and  $\sigma$ 1C). AP-2 has two  $\alpha$  isoforms ( $\alpha$ 1 and  $\alpha$ 2). AP-3 has two  $\beta$  isoforms ( $\beta$ 3A and  $\beta$ 3B), two of  $\mu$  ( $\mu$ 3A and  $\mu$ 3B) and two of  $\sigma$  ( $\sigma$ 3A,  $\sigma$ 3B). The large subunits of each of the complexes (except AP-5) have characteristic "ear" domains on their C-terminal ends preceded by the "hinge" domain, which connect the ears to the core complex (Figure 21 B). AP-1, AP-2 and AP-3 hinge domains possess clathrin binding motifs which aid the complex to bind and interact with the terminal domain of the clathrin heavy chain to form the clathrin-coated vesicles (CCVs). While the ears are responsible for the interaction with regulatory/ accessory proteins, the core subunits  $\mu$  and  $\sigma$ , recognize motifs such as the tyrosine-based YXX $\Phi$  and di-leucine-based [D/E]XXXL[L/I] in the cytoplasmic domain of transmembrane cargo proteins. Within the motifs, X represents any amino acid and  $\Phi$  represents any bulky hydrophobic amino acid such as leucine, isoleucine, methionine, valine or phenylalanine. These motifs are recognized by AP-1, AP-2 and AP-3 and in particular the tyrosine-based YXX $\Phi$  is recognized by  $\mu$ 1- $\mu$ 3 and the dileucine-based motif by the combinations  $\gamma$ - $\sigma$ 1,  $\alpha$ - $\sigma$ 2 and  $\delta$ - $\sigma$ 3 (Mattera, R, et al., 2011). By way of combination of the different subunit isoforms, the different formed AP heterotetramers have unique sites of expression and function. For example, AP-1A (having  $\mu$ 1A) is ubiquitously expressed, AP-1B (with  $\mu$ 1B) is epithelium specific, AP-3A ( $\delta$ ,  $\beta$ 3A,  $\mu$ 3A, and  $\sigma$ 3) is ubiquitous and AP-3B ( $\delta$ ,  $\beta$ 3B,  $\mu$ 3B, and  $\sigma$ 3) is neuron specific (Simpson, F. et al., 1997; Zizioli, D. et al., 2010). However at the cellular level, the localization is well established. AP-1 and AP-4 are found at the TGN, AP-2 at the

plasma membrane, and AP-3 and also AP-4 function in the endo-lysosomal pathway (Hirst, J. et al., 2012). AP3 localises to early endosome associated tubules, where AP1 is also detected (Peden, A.A., et al., 2004). AP1 is responsible for the transport of lysosomal hydrolases between the TGN and endosomes. AP2 associates with the plasma membrane and is responsible for endocytosis. AP3 is responsible for protein trafficking to lysosomes and other related organelles, such as LRO. AP4 and AP5 are less well characterised. However, AP4 is has also been shown to play a role in TGN to endosome transport and in basolateral sorting in polarised cells. AP5 has been shown to play a role in neuronal development and homeostasis similar to AP4, suggesting that it could share funcational relationship with AP4 in vesicular trafficking (Hirst, J. et al., 2012).



**Figure 21. Adaptor Complexes.** A) AP-1 and AP-3 are found localized in the tubular sorting endosomal compartment (Theos, A.C. et al, 2005; Peden, A.A. et al., 2004). AP-3 is involved in cargo transport from the tubular endosome to lates endosomes and lysosomes. AP-1 is also present at the TGN and mediates transport between TGN and the endosomes. AP-2 stimulates clathrin mediated endocytosis at the plasma membrane. AP4 transports cargo between the TGN and endosomes (Hirst, J. et al., 2011). B) Structures of the different hetero-tetramers AP1-5 (Park, S.Y. and Guo, X., 2014).

The importance of the adaptor complexes in intracellular protein transport and hence their physiological roles is discerned from the variety of inherited diseases and pathological states that result from the dysfunction of these proteins and also based on experiments on knock-out mouse models. Knockout of  $\gamma 1$  and  $\mu 1A$  has shown the importance of AP1 in embryonic development (Meyer, C. et al., 2000; Zizioli, D. et al., 1999). AP-2 knock-out in the mouse model showed it is also essential for embryonic development (Mitsunari, T. et al., 2005). Missense mutations in  $\sigma 2$  cause familial hypocalciuric hyper calcaemia type3 (FHH3), a condition which leads to imbalanced extracellular calcium homeostasis, affecting the parathyroids, the kidneys and the bones. The mutation on Arg15 of  $\sigma 2$  results in defective

endocytosis of a Ca2<sup>+</sup> sensing receptor (CaSR), probably because of a disturbed interaction of the mutated  $\sigma^2$  with the dileucine motif on the cytoplasmic tail of CaSR (Nesbit, M.A. et al., 2013). Mutations in AP-3 ( $\beta$ 3A) cause Hermansky-Pudlak syndrome type-2 (HPS-2) in humans, a disorder characterized by defective lysosome related organelles and hence at the tissue level leads to oculocutaneous albinism and bleeding problems with platelet abnormalities (Dell'Angelica, D.C. et al., 1999). AP-4 when mutated in each of the four subunits leads to hereditary spastic paraplegia (HSP) a genetic disorder, which leads to lower extremity spasticity and weakness, with or without neurologic abnormalities (Jamra, R.A. et al., 2011).  $\zeta$  subunit mutations revealed the role of AP-5 in HSP as well (Slabicki, M., et al., 2011). This led to the understand both AP-4 and AP-5 may be involved in sorting similar cargo or both being important for axonal maintenance (Hirst, J. et al., 2013)

#### 5.2.1 AP-1

AP-1 localises at the TGN and recycling tubular endosomes. Via the core unit, the complex interacts directly with Arf-1 (via the  $\gamma$  and  $\beta$ 1 subunits) and the phosphoinositol PI(4)P (via the y subunit ) (Zhu, Y. et al., 1999; Wang, Y.G. et al., 2003; Ren, X. et al., 2013). The Arf1mediated membrane recruitment of AP-1 was first demonstrated by its sensitivity to Arf-GEF inhibitor Brefeldin-A (Park, S.Y. and Guo, X. 2014). AP1 was shown to mediate secretory granule biogenesis and bidirectional transport between the TGN and endosomes (Burgess, J. et al., 2011; Stamnes, M.A. et al., 1993). In epithelial cells, AP-1B was shown to interact with phosphatidyl 3,4,5- inositol trisphosphate (PI(3,4,5)P3) (Fields, I.C. et al., 2010) and Arf6 (Shteyn, E. et al., 2011), to localise at the recycling endosomes (REs), and participate in basolateral sorting in polarized epithelial cells (Folsch, H. et al., 1999). AP-1A was shown to be essential for sorting cargo from the TGN to the basolateral plasma membrane and AP1B transports cargo from common recycling endosomes to the basolateral basolateral plasma membrane (Gravotta, D. et al., 2012). Therefore, both AP-1A and AP-1B are essential to maintain cell polarity by mediating polarized sorting within the secretory and endocytic pathways (Bonifacino, J.S. 2014). The same was demonstrated in neurons where AP-1A disruption led to missorting of somatodendritic proteins such as NR2A and NR2B to the axon (Farias, G.G. et al., 2012). In Arabidopsis it was shown that AP1 plays a role in cytokinesis using AP1m2 loss of function mutant. Cytokinesis specific syntaxin KNOLLE (KN) was mislocalised and aggregated around the division plane in the AP1m2 mutant (Teh, O.K. et al., 2013).



**Figure 22. Structural Conformations of AP-1** (Park, S.Y. and Guo, X., 2014). The architecture of the AP-1 complex and the corresponding structural models show the locked and open states of the protein in the absence and presence of Arf1, respectively. In the locked state, the core cargo-binding site is masked by the large  $\gamma$  and  $\beta$  subunits. Upon interaction with Arf1, the complex opens up to reveal the YXX $\Phi$  and [DE]XXXL[L/I] binding sites (yellow circles).

## **5.3 Epsins**

Among all the accessory molecules participating to CCV formation, epsin proteins interacts directly with the AP complexes and participate to the initial membrane curvature crucial for clathrin vesicle budding and also signalling. They belong to a class of endocytic adaptors and play an important role in clathrin mediated endocytosis and also signalling (Sen, A. et al., 2012). Epsins have been described to display essential physiological roles such as embryonic development in metazoans (Chen H et al., 2009) and cell viability in yeast (Wendland, B. et al., 1999). Across the eukaryotic phylum, only a handful of epsin paralogs, including the classical epsins: epsin-1 (Chen, H. et al., 1999), epsin-2 (Rosenthal, J.A. et al., 1999), epsin-3 (Spradling, K.D. et al., 2001) and epsin-related protein (Eps-R), have been identified (Duncan, M.C. et al, 2003). Epsin-1 and epsin-2 participate in clathrin mediated endocytosis at the plasma membrane. Epsin-3 was described as a unique epsin found only in wounded epithelia. The protein localises to the nuclear periphery and was suggested to play a role in activating keratinocyte migration during tissue morphogenesis (Spradling, K.D. et al.,

2001). Likewise, Epsin15 was shown to interact with AP2 and aid clathrin mediated endocytosis (Chen, H. et al., 1999). On the other hand, epsinR/enthoprotin/CLINT (Chidambaram, S. et al., 2003) stimulates AP1/clathrin-mediated budding events at the TGN and directly interacts with PI(4)P, clathrin and the  $\gamma$  ear appendage of AP1. These proteins bind to specific phospholipids via their ENTH domain (Ford, M.G.J et al., 2002) and induce local membrane curvature and thus aid CCV formation.



Figure 23 AP-1 interaction with the ENTH domain containing Epsin (adapted from Guillemin, V.L. et al., 2004). The clathrin adaptors AP-1 complex and GGA2 are targeted to the membrane via interactions with active Arf1 (GTP-bound) and sorting motifs present in the cargos. Both recruit clathrin triskelia via clathrin binding regions present in the hinge region. The ear domain of GGA2 and the AP $\gamma$ 1 subunit interacts with ENTH-domain containing enthoprotin and epsinR, respectively. The ENTH domain of these proteins interacts with phosphoinositols present in the lipid bilayer, while the LFDLM domain contributes to clathrin assembly.

## 5.4 Rabs

Rab proteins are small GTPases belonging to the Ras-like GTPase superfamily and regulate various steps of vesicular and membrane trafficking, right from formation of transport carriers from the donor membrane and movement of carriers along cytoskeletal tracks to anchoring and fusion at the correct acceptor membrane (Stenmark, H. 2009; Ligeti, E. et al., 2011). There are more than 70 Rabs that have been identified in humans and localized to the membranes of various intracellular organelles. The association with membranes is mediated by hydrophobic geranyl groups that are attached to the

carboxy terminal cysteine residues (one, or two in many cases) of the Rabs. Rabs by interacting with coat components, molecular motors and also SNAREs carry out their vital role of membrane traffic regulation. Rab proteins function as molecular switches that alternate between an "active" GTP bound state and an "inactive" GDP bound state. While the switch from GDP to GTP bound states is catalyzed by guanine nucleotide exchange factors (GEFs), the reversal of GTP to GDP by hydrolysis is catalyzed not only by the intrinsic GTPase activity of the respective Rab itself but also by GTPase activating proteins (GAPs). There are also factors that can maintain the Rabs in their GDP bound inactive state and they are called the GDP dissociation inhibitors (GDIs) (Matsui, Y. et al, 1990). RabGDIs were shown to chaperone geranylgeranlyated Rab GTPases in the cytosol (Ullrich, O. et al., 1993). The association of Rab GTPases to distinct membranes of compartments is also influenced by the membrane bound GDI displacement factors (GDFs) which recognize the specific RabGDIs and remove them so the geranlgeranlyated Rab GTPase can associate with the appropriate membrane (Sivars, U. et al., 2003).

In the vesicular budding process, although different Rabs have been found to play profound roles, Rab5 is crucial in cargo sequestration. Working together with its GDI, Rab5 was found to be essential for assembling clathrin coated pits at the plasma membrane and also clathrin mediated endocytosis of transferrin receptors (McLauchlan, H. et al., 1998). Rab5 was also found to be involved in the AP2 uncoating process by working together with its GEF, GAPVD1 (GTPase activating protein and VPS9 domain-containg protein 1) and promoting dephosphorylation of AP $\mu$ 2 and increase of PI(4,5)P<sub>2</sub> turnover (Semerdjieva, S et al., 2008). As for vesicle motility, Rab27A is a good example. Rab27A recruits the adaptor melanophilin to the melanosome membrane and thereby connect the melanosome to the myosin motor MyoVa. MyoVa shuttles the Rab27A positive melanosomes to the cell periphery, a crucial event for proper functioning of melanocytes (Menasche, G.et al., 2006). In tethering and fusion processes, again Rab5 serves as a wonderful example. Rab 5 interacts in its active form with Rabenosyn5 and early endosomal antigen1 (EEA1). Rabenosyn 5 interacts with VPS45, a member of the Sec1 family of SNARE regulators. Together with EEA1, Rabenosyn 5 also interacts directly with the endosomal SNAREs, syntaxin 6, syntaxin13 and syntaxin 7 and thereby regulate docking and fusion of the early endosome (Stenmark, H., 2009). The molecules mentioned in the above mentioned trafficking processes can be clearly related to the depiction on (Figure 24B).



**Figure 24. A) Rab activation cycle.** The GDP bound inactive Rab is converted to the GTP bound active Rab by the activity of a guanine nucleotide exchange factor –GEF and vice versa by a GTPase-activating protein – GAP, with the release of an inorganic phosphate by hydrolysis. The naïve Rab in GDP bound form is presented by a Rab escort protein (REP) to a geranylgeranly transferase which geranylates the Rab on its C terminal cysteine residues. The geranylgeranlyated Rab-GDP is recognized by the Rab GDP dissociation inhibitor (GDI) which regulates the membrane association of the Rab. Interaction with a membrane bound RabGDI displacement factor (RabGDF) targets the Rab to specific membranes. B) Rab co-ordination in vesicular trafficking. Vesicular budding is mediated by GTP-bound active Rab, which interacts with a sorting adaptor. Next, PI (phosphatidyl inositol) kinases or phosphatases are recruited and the PI is modified, leading to uncoating. RabGTPases can drive vesicle tethering is ensured by recruitment of tethering factors which interact with SNAREs leading to membrane fusion. C) Localisation of selected Rab GTPases and vesicle trafficking routes in an epithelial cell (adapted from Stenmark, H. 2009).

#### 5.5 Rab11

In mammals there are three isoforms of Rab11: Rab11A, B and Rab25, and they share high sequence identity (Welz, T. et al., 2014). The expression of Rab11A is ubiquitous, but Rab11B is restricted to the brain, testes and heart (Lai, F. et al., 1994), and Rab25 to the lung, kidney and gastric tract (Goldenring, J.R. et al., 1993). Like many other Rabs, Rab11A association to the membrane is mediated by the geranylgeranylation of the C terminal cysteine (CC) residues. Rab11 localises to the TGN, to post-Golgi secretory vesicles and recycling endosomes (Urbe, S. et al 1993). Rab11 is one of the most widely studied RabGTPase and known to be involved in several functional roles in intracellular protein trafficking such as delivery of proteins at cell-cell junctions, secretion of growth factors and cytokines at the plasma membrane, recycling of transmembrane proteins, such as the transferrin receptor and integrins during cell motility (Guichard, A. et al 2014). Indeed, Rab11 regulates the transport of many receptors and adhesion molecules, such as  $\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid(AMPA) receptor, rhodopsin, epidermal growth factor (EGF) receptor, Toll like receptor 4 (TLR4), E-cadherin and N-cadherin (Kelly, E.E. et al., 2012).



**Figure 25 Rab11 distribution and functions** (Welz, T. et al., 2014). As described above, the localization of Rab11 (in green) at the TGN, post-Golgi secretory vesicles and recycling endosomes is depicted in this image. In the recycling process of membrane proteins, Rab11 takes part only in the late or slow recycling, which involves a previous transport of internalized material to early endosomes (EE) and late endosome/ multivesicular bodies (LE/MVB), . Rab11 is not involved in the early or fast recycling process, which implies direct recycling of material from early endosomes to plasma membrane. Rab11 transport directions in different cell types and for different functions are illustrated above with green arrows.

In addition, Rab11 regulates vital cellular processes such as ciliogenesis (Knodler, A. et al., 2010), cytokinesis (Hobdy Herson, K.C. et al., 2003), neuritogenesis (Shirane, M. and Nakayama, K.I., 2006) and oogenesis (Dollar, G. et al., 2002; Schuh, M. 2011). The role of Rab11a in cytokinesis is well studied in drosophila and mammalian cells. In mammalian cells, Rab11 and Arf6 together with family of Rab11 interacting proteins, FIP3 and FIP4, have been shown to deliver recycling endosomes to the cleavage furrow and are essential for completion of abscission, the final step of cytokinesis. The cytokinesis failure was a result of depletion of Exo70p, a component of the exocyst complex, which localizes at the cleavage furrow and interacts with Arf6 (Fielding, A.B., et al., 2005). The secretory function mediated by Rab11A is closely associated with the exocyst complex, which is implied in tethering secretory vesicles to the plasma membrane (Heider, M.R. and Munson, M., 2012). The exocyst complex is a hetero octamer made of eight subunits: Sec10, Sec8, Exo84, Sec5, Sec6, Sec3, and Exo70 (Munson, M. and Novick, P., 2006). Sec 15 is an effector of Rab11 (Wu, S. et al., 2005) and the interaction of the two proteins is supposed to be an anchor point for the exocyst assembly at the vesicular side.

#### 5.5.1 Rab11 and motor proteins

Rab11 interacts with several molecular motor protein complexes that involve bidirectional transport along microtubule and actin tracks. Rab11-positive vesicles transport along actin filaments is mediated by its interaction with MyoVb. For instance, the Rab11/Rab11-FIP2 (Rab11 family interacting protein 2)/ MyoVB complex mediates transport of AMPA receptors from recycling endosomes at the base of the spine to postsynaptic membranes (Wang, Z. et al., 2008). Another example is the role of Rab11-FIP3 complex in vesicles transport to the cleavage furrow in a dynein dependent manner during cytokinesis (Horgan, C.P. et al., 2003). When autophagy is induced Rab11 moves from recycling endosomes to autophagosomes and interacts with Hook, a motor adaptor which is a negative regulator of endosome maturation. Hook anchors endosomes to microtubules. It was shown that Rab11 helps fusion of endosomes and autophagosomes by removing Hook from mature late endosomes and inhibiting its homodimerisation (Szatmari, Z. et al., 204).



**Figure 26. Rab11–motor protein complexes** (Welz, T. et al., 2014). Rab11-GTP (green sphere) builds a complex with microtubule '-' end directed dynein via the interaction between the dynein light intermediate chain (DLIC1) and the Rab11 adaptor FIP3. On the contrary, Rab11GDP-protrudin-KIF5 (Kinesin family protein) complex is mictrotubule '+' end directed. Adaptor protein protrudin binds to Rab11 in its GDP bound state (red sphere). Rab11GTP-FIP2-MyoVb slides along actin filaments towards the '+' end.

#### 5.5.2 Rab11 regulators

Like any other Rab GTPase, the function of Rab11 is also regulated by its GAPs and GEFs. Three GAPs have been found so far to activate Rab11 and they belong to the TBC (Tre2, Bub2p, Cdc16p) domain group of proteins. They include TBC1D11, TBC1D15 and Evi5 (the ectopic viral integration site 5 protein homolog) (Fuchs, S. et al., 2007; Zhang, X.M et al., 2005). On the other hand, only one GEF was recently identified for Rab11 in drosophila, a calmodulin-binding protein related to Rab3 GDP/GTP exchange protein (Crag) (Xiong, B. et al., 2012). However the functional mechanisms entailed by these GAPs and GEF are yet to be dissected in detail.

#### 5.5.3 Rab11 in disease

The role of Rab11 in different human diseases has been elucidated by various studies. Rab11c (Rab25) was found to be an oncogene which is overexpressed in breast and ovarian cancers (Cheng,K.W. et al., 2004) and also a tumor suppressor gene in colon cancer (Goldenring, J.R. et al., 2011). Drosophila Rab11 was shown to be essential for cell-cell communication (Ramel, D. et al., 2013) and cell migration, which was directly influenced by its GAP, Evi5 (Laflamme, C. et al., 2012). Rab11 was also shown to be a causative factor in the neurodegenerative disorder Huntigton's disease, by being essential for proper neuronal glucose uptake (Li, X. et al., 2012)

## 5.6 Hook1

The Hook family of proteins has been identified as potential adaptors for the motor protein dynein. In fungal model systems, hook proteins were shown to be required for early endosomal trafficking. Hook proteins are in general made of three conserved regions: a N terminal globular microtubule binding domain, a central coiled coil domain and an uncharacterized C terminal domain supposed to be involved in cargo binding (Walenta, J.H. et al., 2001). Hook1 was first identified in drosophila playing a role in delivery of plasma membrane receptors to late endosomes after endocytosis (Kramer, H. and Phistry, M., 1996; Sunio, A. et al., 1999; Maldonado-Baez, L et al, 2013). Three members of the mammalian Hook family have been so far identified, Hook1, Hook2 and Hook3. Hook 2 localises at the Golgi and centrosomes and is involved in ciliogenesis in human retinal epithelial cells (Szebenyi, G. et al., 2006; Baron Gaillard, C.L. et al., 2011). Hook3 was found to be at the

Golgi as well and in unidentified vesicular structures (Walenta, J.H. et al., 2001). It was proposed that hook 3 was responsible for the turnover of a macrophage scavenger receptor A (SR-A) (Sano, H. et al., 2007). Hook1 was shown to associate with microtubules via its Nterminal domain and to the cargo proteins via its C-terminus, in drosophila (Kramer, H. and Pistry, M.). Hook1 was also found to co-localize with cargos internalized via clathrin independent endocytic (CIE) in tubular recycling endosomes decorated by Rab 22 and Rab11 (Baez, M.L and Donaldson, J.G., 2013) (Figure 27). In another study, hook1 was shown to interact with Rab7, Rab9 and Rab11 by immunoprecipitation studies. Although there was a strong colocalisation by IFA in cells between Hook1 and Rab7, Rab9 and Rab11 were not found to colocalise much with Hook1. The authors suggested that the interaction detected in vitro maybe weak in vivo and it could be mediated by different molecules (Luiro, K et al., 2004). This study thus suggests a prominent role of hook1 in late endocytic organelles by the interaction with Rab7. However, Rab11 is known to mediate fusion events between the recycling endosomes and the TGN. So the interaction between hook1 and Rab11 and also the HOPS complex found in another study (Richardson, S.C. W., 2003) opened a potential broader functional range for this molecule, notably in early and recycling endosomes. Very recently, a study on mammalian hook1 and hook3 revealed the interaction of the hooks with dynein and dynactin and their role as unidirectional ('minus' end directed) dynein-specific adaptors to mediate cargo transport and motility (Olenick, M.A. et al). Finally, another study showed the pathological association of hook proteins in Alzheimer's diseases by finding hook1 and hook3 localisation in tau aggregates and hook2 in glial components within amyloid plaques. Hook3 deficiency slowed endosomal transport and increased β-amyloid production in cultured cells (Herrmann, L. et al., 2015).



**Figure 27. Hook1 and Rab11 in clathrin independent endocytosis (CIE)** (Baez, M.L and Donaldson, J.G., 2013) Representative CIE cargo (red bars) are internalized without the involvement of clathrin and dynamin, but in an Arf6-dependent manner. The endocytic vesicles mature or fuse into Rab5/EEA1/transferrin positive early endosomes. The cargo is then directed to late endosomes or lysosomes for degradation or recycled back to the PM in a Rab11/Rab22 dependent manner. CIE cargos with cytoplasmic motifs (green bars) avoid the early endosome and go directly to recycling endosomes (REs). Hook1 aids recycling of CIE cargo, like CD98 and CD147, via its interaction with microtubules at the level of the recycling endosomes.

# 6. Protein trafficking in T. gondii

As mentioned earlier, *T. gondii* possesses an endoplasmic reticulum (ER) that is contiguous with the nuclear envelope and a Golgi apparatus consisting of a single stack of three to five cisternae. Genome sequencing indicates that the molecular machinery regulating vesicular transport in Apicomplexa is partially conserved compared to other Eukaryotes. Components of the vesicle budding, transport and fusion machinery have been identified, including *N*-ethylmaleimide-sensitive fusion (NSF) factor (Chaturvedi, S. et al., 1999), multiple Rab proteins (Kremer, K. et al., 2013), Soluble N-ethylmaleimide-sensitive-factor Attachment protein Receptor proteins (SNAREs) (Jackson, A.J. et al., 2013), subunits of the coatomer (Pfluger et al., 2005), clathrin adaptor complexes (Ngo et al., 2002), Dynamin

related protein B (DrpB) (Breinich, M.S. et al., 2009), Sortilin Like Receptor (SORTLR) (Sloves, P.J. et al., 2012) and Vacuolar Protein Sorting (VPS) proteins (Sangare, L.O. et al., 2015; Guyot, J.M. et al., 2015). In addition, evolutionarily conserved sorting motifs functioning in the transport of proteins to micronemes and rhoptries have been characterized (Cristina, M.D. et al., 2000; Hoppe, H.C. et al., 2000). Strikingly, *T. gondii* and possibly other apicomplexan parasites use their endolysosomal system as an intermediate track to transport proteins destined for regulated secretion and secretory organelle biogenesis (Tomavo, S., 2014). Likely, merging the endocytic and exocytic pathways allows convenient access to proteases necessary for the maturation of secretory proteins. During cell replication, micronemes and rhoptries are assembled *de novo* and positioned at the apical pole of each daughter cell (Nishi, M. et al., 2008). The assembly is aided by the highly polarized organization of the secretory pathway. Proteins flow in a sequential fashion from the ER to the Golgi apparatus, and from there to endosomal-like compartments, to eventually reach intermediate compartments, precursors of mature rhoptries and micronemes (Soldati, D. et al., 2001).

### 6.1 The *T. gondii* plant-like endosomal system

T. gondii displays a stripped down version of the more complicated vesicular protein trafficking system of mammalian cells and other unicellular Eukaryotes. For instance, Apicomplexa possess only a basic set of Rabs (Kremer, K. et al., 2013). In addition, several highly conserved endocytic factors are missing. Indeed, T. gondii and the other Apicomplexa lack nearly all of the components of the Endosomal Sorting Complexes Required for Transport (ESCRT). T. gondii also lacks the GGAs that regulate protein transport between the TGN and the lysosomes (Tomavo, S. et al., 2013). The spatial organization and the functions of the endosomal and exocytic compartments in T. gondii have for long remained unclear. In mammalian cells, the early endosome (EE) compartment is involved in regulating the transport of endocytosed material to the sorting endosomes for recycling purpose or to the late endosomes for degradation (Mellmann, I., 1996). Early studies indicated that in T. gondii, TgRab5A, which classically defines EE, localises to a tubulo-vesicular compartment closely associated to but distinct from the Golgi, which can be dispersed by Brefeldin A (BFA) and to large, lucent vesicles containing internal membranes (Robibaro, B. et al., 2002). In a similar way, TgRab7, a marker of late endosomes (LE) in mammalian cells, was detected in T. gondii in a restricted compartment adjacent to but distinct from the Golgi apparatus (Parussini, F. et al., 2010). These early observations suggested that T. gondii has diverted the endosomal system activity to a secretory purpose. This hypothesis was recently confirmed by an overexpression screen of the Rabs expressed in T. gondii, showing that TgRab5A and TgRab5C function as important regulators of the traffic to micronemes and rhoptries (further developed in the next chapters). Of note, EEs have not yet been identified in plants (Dettmer, J. et al., 2006). There are strong evidences that plants rather possess a hybrid TGN/EE compartment serving as a sorting platform for protein trafficking to the different subcellular compartments. This hybrid TGN/EE would also be the first site for delivery of endocytosed material (Dettmer, J. et al., 2006; Contento, A.L. and Bassham, D.C., 2012). Thus, T. gondii seems to possess an endosomal-like system more similar to plants than fungi or vertebrates. Importantly, so far, neither clathrin-dependent nor caveolae-regulated endocytic activities have been demonstrated in T. gondii. Recently, Meissner and co-workers examined this aspect by characterizing the function of clathrin (Pieperhoff, M.S. et al., 2013). They found that clathrin localized to the Golgi apparatus, but not at the parasite surface. In addition, functional ablation of clathrin resulted in Golgi aberrations, a block in the biogenesis of the microneme and rhoptry organelles, and of the pellicle, suggesting a major role for clathrin in the secretory pathway but not in endocytosis. However, due to the lack of traceable endocytic markers, this conclusion has not yet been clearly ascertained and requires further investigation. Interestingly, V. Carruthers and colleagues have recently observed the uptake of large proteins from the host cell into intracellular parasites after depletion of the cathepsin L protease (Dou, Z. et al., 2014). Thus, this could represent a useful tool to test the function of clathrin in endocytosis. An additional intracellular compartment distinct from the other commonly known apical organelles has been recently identified in T. gondii. This compartment displays similarities to the digestive vacuole of plants and therefore was called the plant-like vacuole (PLV or VAC) (Parussini, F. et al., 2010; Miranda, K. et al., 2010). Given its location proximal to the LE marked by TgRab7 and TgVP1 (vacuolar proton pump) and the presence of cathepsins, the authors reasoned that the VAC is the T. gondii equivalent of a lysosome or lytic vacuole in yeast. However, the putative role of the VAC in the degradation of ingested host cell material by the parasite still needs to be investigated.


Figure 28. T. gondii utilizes its endosomal system to a secretory purpose

The early (EE: Rab5) and the late (LE: Rab7) endosomal compartments are localized in close proximity to the Golgi apparatus and are involved in MIC and ROP biogenesis. A conversion from EE to LE endosomes has never been investigated in *T. gondii* as previously described in mammalian cells. The recycling compartment, containing Rab11 in other Eukaryotes, regulates the secretion of newly synthesized Inner Membrane Complex (IMC) proteins from the TGN (Rab11B) or the endosomal compartments (Rab11A) to the forming buds of daughter cells and to the plasma membrane (PM), respectively. The digestive vacuole (VAC) shows alocalization proximal to the late endosomes marked by Rab7 and contains the cathepsin-like protease CPL.The VAC is required for MIC processing. Its role in the degradation of ingested material from the parasite plasma membrane has not yet been investigated.

#### 6.2. T.gondii Rab11

The function and localization of  $T_g$ Rab11A and  $T_g$ Rab11B, which are important regulators of the recycling activity in other Eukaryotes, were characterized *in T. gondii*. The role of Rab11A in host cell invasion and replication of apicomplexans has been only partially elucidated and the molecular mechanisms regulating these processes remain unknown. Rab11A was first mentioned to be rhoptry associated (Bradley, P.J. et al., 2005). Subsequently, using an inducible over-expression strategy using the ddFKBP system, wherein Rab11A, N terminally tagged with cmyc and mcherry, was over-expressed under the control of a destabilization domain (DD) (Herm-Gotz, A. et al., 2008; Agop-Nersesian, C. et al., 2009). This work led to the conclusion that Rab11A partially localizes to the rhoptries and with M2AP, a marker for the endosome-like compartments (ELC). Furthermore, the authors concluded that overexpression of a mutated (GTPase domain – N126I) inactive form of the protein did not affect apical secretory organelles (micronemes, rhoptries), nor dense granules (DG) biogenesis. The only defects were demonstrated to be in the recruitment of the late glideosome components to the IMC, leading to a cytokinesis defect. Rab11A was also shown to be involved in the constitutive secretion of the surface antigen SAG1 at the parasite plasma membrane.

 $T_g$ Rab11B localizes to the Golgi at the initial phase of cell division and accumulates to the nascent IMC of daughter parasites. Overexpression of  $T_g$ Rab11B mutated in its GTPase domain impaired daughter cell budding because of impaired IMC biogenesis (Agop-Nersesian, C. et al., 2010). However this did not affect formation of the sub-pellicular microtubules or the conoid, showing that they are both independent processes. These results demonstrate a role for Rab11B in the trafficking of newly synthesized IMC proteins from the Golgi to the daughter cell buds. These two studies also thus indicate that the recycling compartment, such as the early and late endosomes, might have been repurposed to function in the secretory pathway.

#### **6.3** Microneme protein trafficking in the secretory pathway

A recent study showed the difference in the secretory modes of microneme proteins and their functions directly correlate with distinct trafficking pathways during de novo MIC organelles biogenesis. Following an overexpression screen of Apicomplexa Rabs, T. gondii Rab5A and Rab5C were identified as key regulators of only a subset of MIC proteins. Upon overexpression of dominant negative TgRab5A, MIC3, MIC8 and MIC 11 were re-routed in the constitutive pathway and released to the vacuolar space. This was not the case for MIC2, AMA1 and M2AP, which reach their final microneme destination. Furthermore, initially thought to be a conglomerate of proteins within the same organelle, the sequential secretion and different functions of MIC proteins was a puzzling issue. Two-color STED (Stimulated-Emission-Depletion) measurements were used to finely pinpoint the location of different microneme proteins and it was found that MIC2 and MIC3 were detected in two distinct microneme sub-populations (Kremer, K. et al., 2013) Therefore, this study demonstrated that microneme proteins consist of at least two independent populations, with distinct transport pathways correlating with distinct subcellular localization and secretion kinetics. In a later study, it was shown that T. gondii cathepsin L-like protease (TgCPL), which localized in the VAC compartment, is required for the processing of microneme protein precursors,

proTgM2AP and proTgMIC3, in a pH-dependent manner (Parussini, F. et al., 2010). Unlike most eukaryotic cells where the TGN functions as the site of biogenesis of regulated secretory organelles, the authors proposed a model by which the micronemes are generated from the endocytic system, in particular the LE or the VAC, where the MIC pro-proteins get processed (Parussini, F. et al., 2010). It is thus intriguing that TgRab7 dominant negative mutants were found to not display any defect in MIC processing and transport to their final organelle destination (Kremer, K. et al., 2013). Thus despite the close proximity observed between TgRab7-LE and the VAC, no functional relationship can be drawn yet between these two compartments for apical organelle biogenesis.

As mentioned in section 2.4.2.1, microneme proteins generally function in complexes and ideally as a partnering of one transmembrane protein with one or more soluble proteins These complexes are built early in the secretory pathway and the interaction between the different partners is required for their correct trafficking from the TGN to the MIC organelles. MIC proteins are also synthesized individually as immature proteins, which undergo various protein processing events, either during transport in the secretory pathway or after release. These processing steps involve distinct proteases (Parussini, F. et al., 2010). M2AP is transported together with its transmembrane binding partner MIC2, which provides targeting information (tyrosine-based motif) to navigate through the early secretory organelles including the ER and the Golgi apparatus (Huynh, M.H. et al., 2003; Breinich, M.S. et al., 2009). From there on, M2AP pro-peptide functions as a sorting domain and aids the complex through the early to late endosomes to eventually enter the micronemes (Harper, J.M. et al., 2006). Indeed, M2AP, deleted of its pro-peptide, successfully navigates through the ER, the Golgi apparatus and EEs but is arrested in the LEs (Harper, J.M. et al., 2006). In addition, a maturation-resistant mutant of TgM2AP failed to remain stably associated with the adhesive protein TgMIC2 and as a consequence, both proteins were secreted inefficiently and the parasite was less invasive (Harper, J.M. et al., 2006). These data clearly indicated that each component of the complexes possesses distinct but complementary sorting signals to help the complex navigate through the secretory pathway (Figure 29). Trafficking signals within the MIC5 pro-peptide appear to be employed at an earlier stage in the secretory system in comparison to the M2AP pro-peptide since trafficking-defective mutants are diverted before reaching the LEs. This defect correlated with an absence of endosomal proteolytic processing (Gaji,R.Y. et al., 2011). Furthermore, contrary to M2AP, MIC3 pro-peptide mutants are diverted to the parasitophorous vacuolar space, presumably via the dense granules (El Hajj, H. et al., 2008).



Figure 29: Sorting and vesicular transport mechanisms of MIC proteins during organelle biogenesis

MIC proteins navigate through the secretory system as complexes. The scheme depicts the transport of the microneme protein complex MIC2/ M2AP. **1**- Soluble M2AP is recognized by the cargo receptor TgSORTLR, which might interact with the adaptin complex AP1 via its dileucine motif (of note, the role of the dileucine motif in this interaction has still to be demonstrated). Transmembranous MIC2 might associate directly with AP1 via its tyrosine-based (YXXΦ) motif. The interaction between the two members of the complex is also required for the correct sorting of both proteins. The MIC2/ M2AP complex moves out of the Golgi via vesicular budding in a clathrin- (clathrin-coated vesicles: CCV) and DrpB- dependent manner towards the endosomal-like compartments: early endosomes (EE: positive for Rab5) and late endosomes (LE: positive for Rab7 and VP1). **2**- LE might transiently connect with the VAC compartment for the acquisition of the Cathepsin Protease L (CPL). Alternatively, the complex could be directly transported to the VAC compartment by unknown mechanisms. CPL is required for M2AP processing and targeting to the final MIC organelles. This last step seems to require the pro-peptide of M2AP, as a M2AP protein deleted for the pro-peptide accumulates in a compartment positive for VP1. TgSORTLR is recycled back from the endosomal-like compartments to the Golgi apparatus by the retromer complex.

Together, these data, in addition to the report of an extensive interchangeability of microneme propeptides (Gaji, R.Y. et al., 2011), leads us to understand that pro-peptides contribute to a shared trafficking function even though it is at various distinct sites within the secretory

system, with the site of mutant diversion or retention reflecting the earliest propeptidedependent sorting step in the secretory pathway.

## 6.4 Rhoptry protein trafficking in the secretory pathway and the role played by TgAP1

The identified mechanisms of ROP proteins' sorting are very similar to the ones characterized for the MIC proteins. It has been shown that two members of the ROP2 family, ROP2 and ROP4, contain a dileucine (LL) motif and a tyrosine-based sorting signal (YXX) motif), respectively, both signals being known to be recognized by adaptin complexes (Hoppe, H.C. et al., 2000; Ngo, H.M. et al., 2003). Mutating either one of the signals blocks the delivery of the proteins to the mature rhoptries causing them to accumulate in large vesicles resembling multivesicular bodies (MVB), which colocalize with the endosomal marker vacuolar protein sorting 4 (VPS4) (Ngo, H.M. et al., 2003). In addition, it was shown that TgRab5A and TgRab5C activity regulates rhoptries biogenesis, as overexpression of dominant-negative forms of the proteins inhibits ROP formation and ROP proteins were rerouted to the vacuolar space (Kremer, K. et al., 2013). By contrast, a dominant-negative mutant of TgRab7 did not affect ROP biogenesis, suggesting that protein processing and trafficking do not require the late endosomal compartment. However, a recent study showed that depleting Mon1, the putative GEF factor for TgRab7, impairs rhoptry, microneme and dense granule biogenesis, leading to contradictory conclusions on the role of Rab7 in secretory organelles formation (Morlon-Guyot et al., 2015). These findings led to the conclusion that protein targeting to the rhoptries occurs via the endocytic pathway. Keith Joiner and colleagues examined in details the role of the adaptin  $1 \mu$  subunit (AP $\mu$ 1) in rhoptry biogenesis. They showed that the tyrosine-based motif (YEQL) present in ROP2 aids the targeting of the protein to the rhoptries (Hoppe, H.C. et al., 2000). In a subsequent study it was shown that APµ1 localizes to the Golgi/TGN, to Golgi associated vesicles and to both the rhoptry membrane and membranous lumen (Ngo, H.M. et al., 2003). The over-expression of a D176A dominant-negative mutant of APµ1, impaired in the association with the YXX\$\$\$\$ motif, led to disturbed ROP2 sorting and in a more general defect in rhoptry biogenesis characterized by the accumulation of immature rhoptries (Ngo, H.M. et al., 2003). No defect was observed in the formation of dense granules or micronemes. The authors thus concluded that the YXX\$\$\phi\$ motif (Hoppe, H.C. et al., 2000) and also the dileucine motif (Ngo, H.M. et al., 2003) of the supposed cytoplasmic tail of ROP2 aids the sorting of the protein at a postGolgi level. A direct transport of ROPs from the TGN to rhoptries was not favored because the tyrosine-based sorting mutation in ROP2 did not retain the protein in the TGN nor re-route it to the vacuolar space. A later study revealed the absence of the predicted transmembrane domain in ROP2 and demonstrated that the association of ROP2 with the parasitophorous vacuole membrane is mediated by an amphipathic peptide enclosed in the N-terminal region(Labesse, G et al.; 2009).

Although the tyrosine-based sorting motif may explain the targeting of transmembrane ROP proteins, mapping of the domains required for the trafficking of soluble ROP1 revealed that two domains, the ROP1 pro-peptide and a central peptide (Bradley, P.J. and Boothroyd, J.C., 2001; Striepen, B. et al., 2001), were involved in its sorting. Of note, it was shown that the pro-preptide of ROP2 is cleaved in mature rhoptries by the cathepsin B, toxopain-1, suggesting that in contrast to MIC, maturation of at least a subset of ROP proteins occurs late in the secretory pathway (Que, X. et al., 2002). Even though the concept of escorter proteins has not been described within the family of ROPs in T. gondii, this mechanism was described in Plasmodium, where soluble rhoptry proteins RAP2 and RAP3 form an oligomeric complex with transmembrane RAP1 (Baldi et al., 2000). It was also subsequently demonstrated that the presence of dileucine and YXX motifs in ROP proteins and hence the adaptins are not the sole factors responsible for targeting the rhoptry proteins, since ROP4 that lacked both domains, was successfully delivered to the mature rhoptries (Bradley, P.J. et al., 2004). Likewise, another ROP2 family member, ROP18 localizes to the rhoptries despite not possessing the signature motifs (Hajj, E.H. et al., 2007). Indeed, TgSORTLR has been later on identified as a key receptor for ROP protein transport (Sloves, P.J. et al., 2012). Together, these data indicate that such as observed for micronemes, sorting of ROP proteins from the TGN to the early endosomal compartments rely on complementary sorting signals present in the different ROP proteins presumably transported as complexes (Figure 30). Apart from AP1 as a member of the transport machinery regulating ROP protein transport, a dynamin-related protein B (DrpB) was also described as playing a crucial role in ROP and MIC biogenesis. Dynamins are large GTPases that act as mechano-enzymes in vesicle scission (Hinshaw, J. E. et al., 2000). The conditional expression of a dominantnegative DrpB mutant led to a defect not only in micronemes and rhoptries biogenesis but also in dense granules formation (Breinich, M.S. et al., 2009). Surprisingly, DrpB resides in a cytoplasmic compartment close to the Golgi, which breaks up during replication and reforms after assembly of the daughter cells. This observation highlights that most of the trafficking activity within the secretory pathway is tightly correlated to the cell cycle and that molecular links must exist to orchestrate the timing of MIC and ROP biosynthesis with daughter cell budding. Interestingly, a single population of pre-ROP vacuoles has been identified so far, by contrast to the MICs. Electron microscopy (EM) studies revealed that pre-rhoptries resemble secretory granules and develop into mature organelles by condensation and elongation of the neck region towards the conoid of the developing daughter tachyzoites (Dubremetz, J.F., 2007). As studied in *Plasmodium falciparum*, where PfRON4 was found in the pre-rhoptries (Roger, N. et al., 1988) before being transported to the mature rhoptry neck, the sub-compartmentalization between ROPs and RONs supposedly takes place via lateral protein sorting and not by fusion of distinct compartments.



## Figure 30. Putative model for sorting and transport mechanisms of ROP proteins during organelle biogenesis

The scheme depicts the sorting and vesicular transport of ROP proteins presented as a complex for the clarity of the model proposed. The mechanisms involved in ROP trafficking are almost similar to the ones identified for the MIC proteins (see Figure 28). However, a major difference would be the lack of the participation of both late endosomes and the VAC compartment in ROP protein trafficking. The ROP might be recognized at the TGN level by AP1 via the tyrosine motif or the DiLeucine motif present in its cytoplasmic tail. ROPs are also recognized by TgSORTLR, which might interact with AP1 via its dileucine motif. TgSORTLR, and tyrosine motif of ROPs are key sorting signals targeting the ROPs to the early endosomes in a clathrin- and DrpB-dependent manner in clathrin-coated vesicles

(CCV). TgSORTLR is recycled back from the endosomal-like compartments to the Golgi apparatus by the retromer complex. Next, the ROP protein complex moves forward to the Pre-rhoptry compartments by unknown mechanisms. However, AP1 might participate in this step, as a dominant-negative mutant of AP1 accumulates immature rhoptries. Finally, the pre-rhoptries mature into rhoptries, a process correlated with processing of the pro-domain present in the ROP proteins.

#### 6.5 TgSORTLR a unique receptor for MIC and ROP protein transport

Recently *T. gondii* sortilin-like receptor, TgSORTLR was identified as an essential cargo receptor which plays a role in the transport of newly synthesized ROP and MIC proteins across the Golgi-endosomal related compartments (Sloves, P.J et al., 2012). Due to the lack of mannose-6-phosphate sorting system, *T. gondii* appears to critically rely on TgSORTLR for secretory protein trafficking. TgSORTLR was demonstrated to localize at the Golgi and proximal vesicles positive for TgRab5 and TgRab7 endosomal markers. Conditional ablation of TgSORTLR disrupts rhoptry and microneme biogenesis, and blocks both invasion into and egress from host cells, and hence also plays a role in host pathogenicity. Upon depletion of TgSORTLR, MIC and ROP proteins were found to be re-routed to the constitutive pathway, thereby being secreted to the vacuolar space or at the plasma membrane of the parasite. Furthermore, the proteins exhibited defects in processing, suggesting that TgSORTLR acts early in the secretory pathway prior to protein maturation. Of note, the study indicates that, unlike yeast and higher Eukaryotes, lower branching Eukaryotes such as *T. gondii*, lack redundant mechanisms for protein trafficking. Even though sortilin is conserved across several species, it was never reported to be an essential protein in other systems.

At the molecular level, TgSORTLR binds to ROP and MIC proteins via luminal N-terminal bladed propeller domains, with analogous mechanisms to those observed in mammalian systems. The cytoplasmic tail of TgSORTLR was demonstrated to recruit the cytosolic sorting machinery involved in anterograde and retrograde protein transport, in particular, the AP1 adaptin ( $\beta$ ,  $\gamma$ , and  $\mu$ 1) and 3 proteins from the retromer complex (Vsp9, Vps26, and Vps35). Importantly, it was also reported that post-translational modifications may be necessary for functional partnering to this cargo receptor since recombinant ROP and MIC proteins produced in bacteria do not interact with the N-terminal domain of TgSORTLR. However, how such a unique receptor differentially recognizes and sorts numerous ROP and MIC proteins to their respective final destination is still unknown. One likely explanation based on the previous studies on ROP and MIC trafficking is that ROP and MIC proteins bind to TgSORTLR in multiprotein complexes with only a subset of proteins forming direct contacts

with the cargo receptor. The authors proposed a model by which, at the time of daughter parasite formation, TgSORTLR binds to rhoptry or microneme proteins and cytosolic cargo sorting proteins such as AP1 at the Golgi (Sloves, P.J et al., 2012). This process would result in a sorting complex, which couples with clathrin-coated vesicles and eventually moves out from external Golgi cisternae. TgSORTLR then further escorts its cargo probably via early endosomes, possibly late endosomes, prior to delivery at their respective destinations. After delivery, the TgSORTLR associates with the retromer complex for retrograde transport and returns to the Golgi for the next cycle of anterograde MIC and ROP transport (Figures 29 and 30).

#### **6.6 Dense granules biogenesis**

Compared to micronemes and rhoptries, the biogenesis of dense granules remains unclear. Unlike higher eukaryotic cells, immature dense granules akin to immature secretory granules have never been observed in *Toxoplasma*. Soluble proteins, if endowed with a signal peptide, are delivered to dense granules before being secreted into the parasitophorous vacuole. However, if supplemented with a GPI signal anchor, they are targeted to the plasma membrane via transport vesicles (Karsten, V. et al., 1998). Furthermore, a mutant version of SAG1 depleted of its GPI anchor signal was directed to the parasitophorous vacuole through the dense granules (Striepen, B. et al., 1998). This led to the conclusion that dense granules constitute the default constitutive secretion pathway for soluble proteins in *T. gondii*.

Due to the similarity of dense granules with the mammalian version of dense core granules, it was inferred that retention and condensation of GRA proteins is the mechanism sustaining dense granule biogenesis. The sorting-by-retention model observed in higher organisms corresponds to the aggregation of the secretory proteins within the granules, preventing them from escaping the maturing organelles by constitutive vesicle budding (Arvan, P. and Castle, D., 1998). The aggregation comes along with changes in the forming granule, such as a mild acidification or a rise in the level of calcium concentration (Chanat, E. and Huttner, W.B., 1991). The dense granules also contain type I transmembrane proteins, found as soluble forms within the organelle (Labruyere, E. et al 1999; Lecordier, L. et al., 1999). The targeting of GRAs to the dense granules is neither pH-dependent nor is it based on signaling motifs present within the protein (Kim, K and Weiss, L.M., 2011). For example GRA4 and GRA7, which possess the signature YXX¢ motif, are not dependent on AP1 for their correct trafficking (Ngo, H.M. et al., 2002). Subsequently, it was shown that GRAs also

form complexes during their transport (GRA2 and GRA5 with GRA3, GRA6, GRA7 with GRA9) (Braun, L. et al., 2008) and that their hydrophobic domains are well concealed, helping them to remain in a soluble form within the dense granules (Gendrin, C.et al., 2008). Upon exocytosis, the proteins would unfold and integrate into the membranous nanotubular network following entry into the parasitophorous vacuole. Alternatively, using GRA5 as a representative member, Gendrin and co-workers demonstrated that transmembrane proteins, which are targeted to the parasitophorous vacuolar membrane (PVM), are sorted through the secretory pathway using a N-terminal domain. This domain prevents the anchoring of the protein to early secretory compartments, allowing it to be lodged only at the PVM (Gendrin, C.et al., 2008). This mechanism might be used by all GRA proteins to be sorted in the constitutive secretory pathway, in contrast to MIC and ROP proteins.

In general, the molecular machinery required to sort and transport newly synthesized GRA proteins from the TGN to the dense granule organelles remain completely unknown and more investigations are needed to clarify this aspect of the secretory pathway.

#### 6.7 Concluding Remarks

The recent findings on ROP and MIC organelle biogenesis helped to characterize the different trafficking routes taken by the newly synthesized proteins and identified new molecules involved in the transport and sorting of the MIC and ROP proteins from the TGN to their final destination. These findings led to the conclusion that *T. gondii* has re-purposed its endosomal system to a secretory function, allowing the MIC and ROP proteins to be efficiently processed along their route towards the apical secretory organelles. However, more studies are needed to provide direct evidence of clathrin-dependent and/or clathrin-independent endocytosis in *T. gondii*. It is possible that *T. gondii* uses all of its endocytic machinery to traffic the proteins destined to the secretory organelles and the IMC. If so, this unique feature could be a result of an evolutionary process to facilitate the parasite's intracellular lifestyle, and therefore might be common to other Apicomplexa.

#### 7. Objectives

Toxoplasma gondii as a powerful invasion machine deploys its unique apical secretory organalles such as micronemes, rhoptries and dense granules in order to invade host cells, replicate within a well protected intracellular niche, manipulate the host cellular functions, egress and perpetuate its intracellular parasitic life cycle. Thus, these apical organelles are of prime importance to the parasite's survival and virulence. Previously work carried out in our lab showed the essential role of the sortilin-like receptor (TgSORTLR) in the biogenesis of micronemes and rhoptries. Conditional ablation of TgSORTLR impaired motility, invasion and egress abilities of the parasite (Sloves, P.J. et al., 2012). Primarily, as a member in the anterograde trafficking pathway, the sortilin-like receptor functions in unison with several other proteins to successfully transport cargo proteins from the TGN to the endosome-like compartment. It was demonstrated in this work that among other functional partners of TgSORTLR the clathrin adaptor complex AP1 subunit  $\mu$  was also present. By homology to what is known in other Eukaryotes, we hypothesized that TgAP1 could participate together with TgSORTLR and other regulatory molecules, in the differential sorting of microneme and rhoptry proteins from the TGN to their distinct target destinations. Notably, previous evidence from literature indicated that TgAP1 does play a role in rhoptry biogenesis (Ngo, H.M. et al., 2002). However the precise trafficking routes regulated by AP1 during ROP biogenesis was not elucidated and we were also interested in finding out whether AP1 could contribute to other parasite functions than ROP and MIC biogenesis.

As a second theme, we looked at the role of the small GTPase Rab11A. Among other aspects of TgAP1 functions, we were interested in its putative role in plasma membrane protein delivery, such as described in other Eukaryotic systems, in particular in plants. Indeed, the mechanisms regulating such activity in *T. gondii* are completely unknown, though being crucial for parasite survival, for example for the delivery of nutrient import system. The functional repertoire of Rab11A is large in mammalian cells, but it was demonstrated that this small GTPase also regulates exocytosis by contributing to the late stages of docking, tethering and fusion of vesicles to the plasma membrane and also to the cleavage furrow of dividing cells (Gidon, A. et al., 2012; Takahashi, S. et al., 2013). In *T. gondii*, Rab11A also affects cytokinesis as well as the secretion of the SAG1 surface antigen at the plasma membrane of the parasite. To date, this GTPase was the only one presenting a putative role in constitutive secretion in *T. gondii*, as the other Rabs, were mostly described as regulating ROP and MIC

biogenesis. Based on these evidences, we decided to investigate the secretory functions of TgRab11A and how it could contribute to parasite invasion and replication.

#### **II-** Materials and Methods

#### II-1 - Cell Culture

#### II-1.1 Culture and maintenance of Host cells and parasites

Throughout this study *Toxoplasma gondii* Type I RH parasites depleted for Ku80 and HXGPRT genes was used unless mentioned otherwise. To generate APm1 knock out parasites by the CreLOX strategy, a special strain of RHDiCreΔKu80ΔHXGPRT (a kind gift from Dr Markus Meissner) expressing the dimerizable Cre subunits was used. Parasites were grown on confluent Human Foreskin Fibroblast (HFF) cells, which were cultured in complete DMEM GlutaMAX-1 (GibcoLife Technologies) supplemented with 10% Fetal Bovine Serum (GibcoLife Technologies) and 1% Penicillin-Streptomycin (GibcoLife Technologies) under standard cell culture conditions in a 5% CO2 incubator at 37°C. HFFs exhibit growth contact inhibition, rapid and robust growth, and well spread out morphology making them the cell line of choice. Passaging cells was done by using trypsinisation with 0.25% Trypsin EDTA (Gibco).

The parasites were allowed to grow by serial passages in the HFFs. Growth was monitored by checking for the formation of parasitophorous vacuoles containing dividing parasites until complete lysis of the HFF monolayer was achieved (2-3 days). Fully egressed parasites were then passed into a new T25 flask containing fully grown HFFs.. In case of experimental usage demanding HFF mechanical lysis, infected host cells were scraped with a sterile cell scraper, followed by passing through a syringe and needles with varying gauge sizes. Parasites were then filtered using a  $3\mu$ m whatman filter unit to be separated from host cell debris. In most cases parasites were centrifuged at 2200 rpm for 10minutes at room temperature or 4°C if necessary. 20µl of parasite suspension was placed on Malassez chamber and then counted once settled down.

#### **II-2. Molecular Biology**

#### **II-2.1 Genomic DNA extraction**

Genomic DNA was isolated from the Type I RH $\Delta$ Ku80 strain parasites using the Promega Wizard genomic DNA purification kit. A pellet of freshly egressed parasites was resuspended in 200 µl Nuclei Lysis solution with 1µl RNase added to it and incubated at 37°C for 20

minutes. Next, at room temperature protein precipitation solution was added, the mix was vortexed briefly and incubated on ice for 5 minutes. The solution was centrifuged at 14000 rpm for 10 minutes. The supernatant was transferred to a fresh eppendorf tube containing isopropanol in order to precipitate the DNA by incubating at -20°C for one hour. The solution was centrifuged at 14000rpm for 30 minutes at 4°C. The supernatant was discarded and the pellet of genomic DNA was washed in 70% ethanol and centrifuged at room temperature for 10 minutes. The ethanol was then discarded and any trace of ethanol was allowed to evaporate completely at room temperature. The genomic DNA was suspended in 40µl of Milli Q water and allowed to dissolve for 15 minutes at 65°C. The DNA concentration was measured in nanodrop spectrophotometer (GE Healthcare) and stored at -20°C for future use.

#### II-2.2 cDNA preparation by RT-PCR

Purified tachyzoites were lysed in lysis buffer without any RNase [1% SDS, 50 mM Sodium acetate, pH 5.2, 10 mM EDTA]. An equal volume of phenol was added to the lysate and incubated for 15 minutes at 65°C with frequent intermittent vortexing steps. Then the solution was subject to centrifugation at 14000 rpm for 5 minutes. The aqueous phase was transferred to fresh an eppendorf tube and subjected once again to phenol extraction. The supernatant was then mixed with 2 times the volume of chloroform and subjected to centrifugation. The aqueous phase which contained total RNA was precipitated using 0.4M Sodium Chloride and 2.5 times the volume of 100% ethanol and incubated overnight at -20°C. The pellet of RNA was obtained after centrifugation for 30 minutes at 14000 rpm at 4°C. The pellet was then washed with 70% ethanol by centrifuging for 15 minutes at room temperature. The pellet was air dried and suspended in MilliQ water. The mRNA concentration was measured and then the purity was checked by performing a PCR to amplify the Super Oxide Dismutase gene (SOD), which in the presence of genomic DNA contaminants reveals a 1.7kb band instead of a 606bp band, by agarose gel electrophoresis. In case of any genomic DNA impurities the sample was treated with 1 unit of RQ1 RNase-free DNase (Promega) per µg of nucleic acid for 30 minutes at 37°C. The total RNA can be purified with another round of phenol-cholorform extraction as mentioned above and the sample was stored at -80°C.

The total RNA was suspended in MilliQ water in the presence of  $10\mu$ M oligoDT. The sample was then incubated sequentially for 2 minutes each at 72°C and then on ice. Reverse Transcription reaction was set using AMV RT (Avian Myeloblastis Virus Reverse Transcriptase, Roche) in presence of RNase inhibitor and 25mM dNTP for 1 hour at 42°C

Following treatment on ice for 10 minutes cDNA was extracted by phenol chloroform extraction and then precipitated. The cDNA was suspended in Milli Q water and checked once again for the purity by qualitative PCR using SOD primers, which should now yield only a 600bp fragment.

#### **II-II.3** Genetic Engineering

#### List of Primers used in our study:

Plasmid	Primers (F: Forward; R: reverse)	Cloning	Linearization
		restriction	enzyme
		sites	
pLic EPSL-cmyc	F:tacttccaatccaatttaatgcCCTCGTTCTCTCCTTCTCA		Ncol
(HXGPRT)	GACGTT		
	R:tcctccacttccaattttagcGAACCCCGTCGTAGCAGGA		
	GAT		
pLicAP1µ-HA	F:tacttccaatccaatttaatgcGGATCTTCCCTAGTTCGCG		SnaBl
(DHFR)	CCAGTCAC		
	R:tcctccacttccaattttagcGGAGAGTCTCAGTTGGTAC		
	TCTCCATTTTGAGT		
pLic Rab5-YFP	F:tacttccaatccaatttaatgcACTTTTGCCTCCACATGCA		Eco47III
(DHFR)	CACC		
	R:tcctccacttccaattttagcGTGAGTGTCTCAGAAGGGA		
	AGAACG		
pLic AP1σ-HA	F:tacttccaatccaatttaatgcGTGATCCACCACTTTGTCG		EcoRV
(DHFR)	AGATCTTGG		
	R:tcctccacttccaattttagcGTCATGTAAGCTTGACTCC		
	ACCTTTAGTGTTGCTC		
DDcMycAP1µ	F:GCGCatgcatATGGCGGGGGGCGTCTGCG	Nsil	
(HXGPRT)	R:GCGCttaattaaCTAGGAGAGTCTCAGTTGGTACTC	Pacl	
	TCCATTTTGAGTGATG		

CreLOXAP1µ-HA	3' UTR		
(HXGPRT)	F:GCGCatgcatATGGCGGGGGGGCGTCTGCG	Sacl	
	R: GCGCgagctcACGGAGAAGGAACGAGGAGCAAAG	Sacl	
CreLOXAP1µ-HA	Coding		
(HXGPRT)	F: GCGCcctaggATGGCGGGGGGCGTCTGCGGTGT	Avrll	
	R:GCGCagatctCTAAGCGTAATCTGGAACATCGTATG	BgIII	
	GGTAGGAGAGTCTCAGTTGGTACTCTCCA		
CreLOXAP1µ-HA	5'UTR		
(HXGPRT)	F:GCGCggtaccCAAGTTCCCGTTTGTCCTGG	Kpnl	
	R:GCGCgggcccTCTTGGGACTGCAAGATCGACTG	Apal	
GST-AP1βear	F:ggatccGAGAACTCCTCTGCCGACAAGGACGTTTTC	BamHI	
	AGA	EcoRI	
	R:gaattcTCACGACCGTGGCGTCAGCC		
GST-AP1γear	F:ggatccTTTCCGCCGATGAATGTCTTGAACGAGGAC	BamHI	
	G	EcoRI	
	R:gaattcTCACGCGAGGAGTCCCGCGG		
GST-Rab11A	F:CGGggatccGAACAAAAACTCATCTCAGAAGAGGA	BamHI	
	TCTGATGGCGGCTAAAGATGAATACTACG		
	R:gcggccgcTCAGGCGGAACAGCAGCCAC	Notl	
pLIC HOOK-HA	F:tacttccaatccaatttagcGCAAAGATGACATGGCGAA		BstBl
(DHFR)	GCAGATGATG		
	R:tcctccacttccaattttagcCGCCTCCCGAGGTGTGACA		
	GAATC		

#### **II-2.4 Cloning methods**

Genomic DNA or cDNA isolated from the Type I RH∆Ku80 strain parasites as detailed above, were used as templates for all PCRs. PCR reactions were carried out using the NEB

Phusion polymerase kit using the High Fidelity buffer and respective primers for each reaction with appropriate annealing temperatures and elongation times. Overall, the standard conditions set for PCRs were as follows,

Initial Denaturation	n 95°C	5m	
Denaturation	95°C	30s	
Annealing	Primer dependant (~50-65°C	) 30s	25-30 cycles
Elongation	72°C	30s/kb	
Final elongation	72°C	10m	
Storage	4°C	$\infty$	

PCR amplifications and all subsequent DNA analysis steps were verified by agarose gel electrophoresis. Agarose (EUROMEDEX) gels of different concentrations were cast in 1X TAE (Tris-acetate-EDTA) buffer, according to expected molecular weights of products. Visualization was achieved using Ethidium bromide (EtBr) at a concentration of  $0.5\mu$ g/ml and images were captured using GelDoc (Biorad). Following PCR amplification or restriction digestion, DNA fragments were purified using NucleoSpin Gel and PCR Cleanup kit (Machery Nagel).

Digested plasmids were dephosphorylated using Fast Alkaline Phosphatase (Thermo Scientific) according to recommended conditions before setting up ligation reactions with the respective insert DNA fragments. TOP10 competent bacterial cells were first thawed on ice for 15 minutes. The DNA mix was then added and bacteria were subjected to consecutive cold (15minutes on ice) and heat shock (45 seconds on 42°C dry bath) steps, followed by incubation on ice for 5 minutes. The transformed bacteria was grown for one hour in 500µl LB medium before being spread onto LB agar plates with ampicillin (unless mentioned otherwise) and allowed to grow overnight at 37°C.

Colonies were screened by colony PCR using DreamTaq Green DNA polymerase kit (Thermos scientific) and appropriate set of primers. Positive colonies were further verified by restriction digestion of the plasmids or by sequencing (Sanger sequencing done at GenoScreen, Institut Pasteur de Lille) or both. If necessary, a "midi prep" of the plasmids was done using NucleoBond<sup>®</sup> Xtra Midi / Maxi kit (Machery Nagel).

#### II-2.5 Schemes describing the different molecular cloning strategies used in our project

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#### ddFKBP inducible over-expression system

Regulated over-expression of Rab11A and AP1 $\mu$  was achieved using the ddFKBP also called DD system. The plasmids coding for both Rab11A<sub>WT</sub> and Rab11A<sub>DN</sub> (containing the point mutation N126I in the GTPase domain) under the DD system are kind gifts from Dr Markus Meissner. We used this plasmid as a template to generate the DDAP1<sub>DN</sub> construct. Briefly the gene of interest (AP1) was placed under the influence of the alpha tubulin based p5RT70 promoter and the DD destabilization domain, which is followed by a cmyc epitope tag alone or together with a fluorescent mCherry tag. The plasmid when transfected, integrates randomly into the genome. Following MPA (mycophenolic acid) and Xanthine drug selection against the HXGPRT selection marker, parasites were cloned. In the absence of Shield-1, the recombinant protein is targeted to the proteolytic degradation pathway. When the synthetic ligand Shield-1 is added the protein expression is stabilized and accumulated in the parasites over time and in a Shield-1dose-dependent manner.



#### CreLOX-based inducible knock-out

The rapamycin induced CreLOX strategy is described in Chapter II, within the Figure 3 of the submitted paper on the AP1 study.

#### Endogenous gene tagging using the pLIC system

We used the pLIC strategy to endogenously tag the genes of interest. The strategy involves amplifying at least 1 kb of the genomic sequence of interest, that contains a unique restriction

site (not present in the pLIC vector) ideally cutting it in equal halves. The PCR product (gene of interest) amplified using the primers listed above, was ligated into the pLIC plasmid previously linearized with the PacI restriction enzyme (Huynh and Carruthers, 2009). The unique linearization site in the middle of the gene of interest was then used to linearise the pLIC plasmid containing the sequence of interest. Upon DNA transfection, the integration into the genome is achieved by a single homologous recombination event in the RH $\Delta$ Ku80 parasite strain.



#### **II.2.6** Transfection

As a standard condition,  $50\mu$ g of non-linearised plasmids was used to transfect  $5*10^6$  freshly egressed tachyzoites in both cases, the DD stabilised overexpresssion system and the CreLOX system. Transient transfections were performed in  $10*10^6$  parasites with  $50 \mu$ g of the following plasmids: HA–tagged *Tg*Rab5A and TgRab7 (V. Carruthers) / cMyc–tagged *Tg*Rab5 and TgRab7 (M. Meissner) / GalNac-YFP (D. Roos); GRASP-RFP (K. Hager) IMC3-cherry (MJ Gubbels), IMC1-cherry (MJ Gubbels), MORN1-cherry (MJ Gubbels), tubulin-YFP (K. Hue) and parasites were allowed to invade HFF cells for 24 h prior analysis. To obtain stable clonal Knock-In parasites, 25  $\mu$ g of plasmids were linearized over-night with the appropriate enzymes, subjected to DNA precipitation by phenol chloroform extraction and then used for transfection. Purified DNA pellets were mixed with parasites suspended in 400 $\mu$ l of Cytomix (120mM KCl; 10mM K<sub>2</sub>HPO<sub>4</sub>/KH<sub>2</sub>PO<sub>4</sub>, pH 7.4; 25mM HEPES pH 7.6; 2mM EGTA pH 7.6; 5mM MgCl<sub>2</sub> ; 0.15mM CaCl<sub>2</sub> ; pH was adjusted to 7.6 with KOH) supplemented with 2mM ATP and 5mM GSH (reduced glutathione) . The mixture was transferred to an electroporation cuvette and pulsed in a BTX Electro Cell Manipulator 600 at

1.5kV/cm, with a capacitance of  $25\mu$ F and resistance of  $50\Omega$ . Transfected parasites were added to a fully confluent T25 flask of HFFs with fresh medium, or in the case of transient transfections inoculated on HFF monolayers grown on coverslips in a 24 well plate. For transiently transfected parasites, following 16–24 hours of growth depending on the goal of the experiment, the cells were fixed with 4% paraformaldehyde and subject to immuno-fluorescence assay (IFA ; detailed below).

#### **II-2.7 Drug Selection**

In case of stable transfections, for plasmids carrying the HXGPRT (hypoxanthinexanthine-guanine phosphoribosyl transferase) cassette as the resistance marker, 24 hours following transfection the parasites were taken under drug selection with mycophenolic acid (MPA) and xanthine (Xan) for a period of 5 days. For plasmids carrying DHFR (Dihydro folate Reductase), the drug Pyrimethamine was added to the growth medium the same day of transfection for a period of 3 days. The parasites that had not been transfected successfully die under drug pressure while the ones containing the resistant cassette survive. The efficiency of transfection rate was assessed by immunofluorescence analysis on the non-clonal parasite population, making use of fluorescent markers or detecting the epitope tag tethered to the gene of interest.

#### **II-2.8** Cloning of transgenic parasites

Once the transfection efficiency was verified the non clonal parasite population was subject to cloning by serial dilution in a 96 well plate containing fully confluent HFFs. It was made sure that each well received only one parasite in a 100µl volume. The plate was incubated for 7 days without disturbance. At the end of the prescribed time duration, the plate was examined under an inverted microscope. Wells containing a single lysis plaque were identified and marked. Such plaques were picked by scraping off the HFF monolayer from the respective wells and transferred to wells of a 24 well plate with fresh medium. Once grown and egressed the parasites were immediately checked by IFA. The positive clones were passed into T25 flasks and continued in cultures for experimental analyses and also frozen using freezing medium (10% DMSO in FBS) as stocks for future use.

#### II-2.9 Transient transfection and Cas9-mediated gene disruption

The plasmid pTOXO\_Cas9-CRISPR corresponds to pUC57 carrying the C-terminally HA/GFP tagged *S. pyogenes Cas9* gene (Cong, L. et al., 2013) fused to 2 nuclear localization sequences expressed under the control of the TUB8 promoter as well as the  $T_g$ U6 promoter driving the gRNA. Twenty mers-oligonucleotides (Forward: GCTGAGCAAAGACAGCCCTC) corresponding to the EpsL gene (TGGT1\_214180) were cloned using Golden Gate strategy (Weber, E., et al. 2011). The ccdB positive-selection marker acts by killing the background of bacterial cells which are not receiving the cloned DNA The plasmid was synthesized and fully sequenced by GenScript (Singapore).

#### **II-3. Cell Biology**

#### II-3.1 Immunofluorescence assays (IFA)

When indicated, infected confluent HFF monolayers were incubated for 1 h with 5 µM of Brefeldin A (Sigma-Aldrich) before fixation with 4 % paraformaldehyde (PFA) in phosphate buffered saline (PBS), for 20 minutes. After quenching with 50mM NH<sub>4</sub>Cl, the coverslips were permeabilised with 0.2% triton or 0.05% Saponin dissolved in 5% FBS-PBS for 30 minutes. The coverslips were then incubated with primary antibodies in 0.1% triton or 0.05% Saponin dissolved in 2%FBS-PBS for 1 h and then washed with PBS, followed by goat antirabbit, goat anti-mouse or goat anti-rat immunoglobulin G secondary antibodies conjugated to Alexa Fluor488 or Alexa Fluor594 or Alexa Fluor647 (Molecular Probes, Invitrogen). Images were acquired using a Zeiss LSM880 confocal microscope. Antibodies used for IFA experiments are the following: rabbit anti-HA (Cell Signaling Technology), mouse anti-cMyc (Thermo Scientific), rat anti-cmyc (Abcam) mouse anti-SAG1 (in house), rabbit anti-GAP45 (D. Soldati-Favre), mouse anti-MIC2 and anti-MIC3 (JF Dubremetz), rabbit anti-M2AP (V. Carruthers), rabbit anti-MIC8 (D. Soldati-Favre), mouse anti-ROP 2-4, mouse anti-ROP5, mouse anti-ROP4, and rabbit anti-ROP1 (all from J.F. Dubremetz), rat anti-VP1 (V. Carruthers), rat anti-TgSORTLR, rabbit anti-tubulin (N.Morrissette) mouse anti-chromo1 (M. Gissot). ProROP4 (G.Ward), ProM2AP (V.Carruthers), ProMIC3 (M. Lebrun), Rab11A (In house), IMC3 (Gubbels, M.J.), IMC1 (Beckers, C.),

#### **II-3.2 Intracellular growth assay**

 $T_gAP1\mu1$ -KO parasites were allowed to invade HFF monolayers for 2h, then treated with 50nM Rapamycin for 6 h. After 3 washes with warm medium, parasites were allowed to grow

for additional 16 h before fixation with 4% PFA. For the DDcMycµ1 and DDcMycmCherry Rab11A strains, parasites were inoculated onto HFF for 2h and treated with or without shield-1 (1µM) for an additional 16 h, before fixation with 4% PFA. In both cases, intracellular parasites were counted after staining with anti-TgGAP45 antibodies. The numbers of parasites per vacuole were counted for more than 200 vacuoles for each condition performed in duplicate. Data are mean values  $\pm$  SEM from three independent biological experiments.

#### **II-3.3 Invasion/Adhesion assay**

Intracellular DDcmycµ1 transfected parasites induced with or without 1µM Shield for 16 h or intracellular  $T_gAP1\mu$ 1-KO parasites induced with 50nM rapamycin as described above were mechanically released from host HFF cells and counted. To study invasion of the Rab11A<sub>DN</sub> mutant, care was taken to limit the induction time with Shield-1 to the minimum as it was performed on freshly egressed extracellular parasites. Hence, 2 hours of induction was decided after verifying the good level of expression of DDmCherrycmycRab11A<sub>WT</sub> and Rab11A<sub>DN</sub> proteins under these conditions. Extracellular Rab11A<sub>WT</sub> and Rab11A<sub>DN</sub> parasites were induced with 0.6µM and 1 µM Shield-1 respectively, for 2hours. Induced parasites were counted and seeded onto HFF monolayers in a 24 well plate at a concentration of  $2*10^6$  parasites/500µl complete medium containing Shield-1 per coverslip. The plate was centrifuged for 2 minutes at 1000rpm at RT to trigger immediate adhesion and synchronized invasion events. Parasites were then shifted at 37°C for 1hour. The slips were then washed with PBS - three times prior to fixation. The cells were fixed in 4% PFA for 10min and then subjected to a red/green invasion assay. Briefly, adherent external parasites were labeled with mouse anti-TgSAG1 antibodies, followed by secondary anti-mouse antibodies coupled to Alexa488, without permeabilisation. After cell permeabilisation with Triton 0.1% for 10 min, invaded intracellular parasites were detected using rabbit anti-TgGAP45 antibodies followed with secondary anti-rabbit antibodies coupled to Alexa594. All the parasites labeled green and green-red, positive for SAG1 were identified as extracellular, while the parasites exclusively red positive for GAP45 staining were considered intracellular. At least, 300 parasites were counted for each condition performed in triplicate. To monitor parasite adhesion activity, the total number of parasites (outside + inside) were counted. Data collected are mean values  $\pm$ standard deviation (SD) from three independent biological experiments.

#### **II-3.4 Moving Junction staining**

Extracellularly Rab11A induced parasites were counted and seeded onto HFF monolayers in a 24 well plate at a concentration of 2\*10<sup>6</sup> parasites/500µl complete medium containing Shield-1 per coverslip. The plate was centrifuged for 2 minutes at 1000rpm at room temperature to trigger immediate adhesion and synchronized invasion events. The plate was immediately shifted to a warm water bath at 37°C and the parasites were fixed with 4% PFA-sucrose at the following time points - 0, 2 and 5minutes. The coverslips were washed with PBS. Adherent or invading parasites were labelled with SAG1 antibody and secondary anti mouse Alexa Fluor 405 antibody sequentially without permeabilisation. After washing with PBS, the parasites were permeabilised with 0.05% saponin for 10 minutes, followed by a blocking step with 5%FBS-PBS for 30 minutes. Next, the parasites were labeled with the moving junction marker, rabbit anti RON4 (M.Lebrun) and secondary anti rabbit Alexa 488. Incubation of antibodies was for one hour each in a humid chamber, and with the secondary antibodies in the dark. After three washes each with PBS following the primary and secondary antibody incubations the parasites were finally labelled with primary rat anti cmyc followed by secondary anti rat Alexa Fluor 594 antibodies. Coverslips were mounted with Mowiol following three washes. Images were acquired using Zeiss LSM880 confocal microscope.

#### II-3.5 Motility (Trail deposition) Assay

3 well glass slides were coated with 100µg/ml BSA-PBS and incubated at 37°C for 1hour. The slides were washed three times with 1XPBS and allowed to dry. Shield-1 induced extracellular DDCMycCherry Rab11A DN or WT parasites were suspended in 1ml HHE buffer (HBSS, 10mM HEPES, 1mM EGTA). 1\*10<sup>6</sup> parasites were seeded per well and incubated for 15minutes at 37°C. Following a single wash with 1X PBS the parasites were fixed with 4% PFA in 1XPBS for 10 minutes at room temperature. Standard IFA protocol was followed wherein only SAG1 was used as the primary antibody diluted in blocking buffer (5% FBS-PBS), followed by goat anti-mouse immunoglobulin G secondary antibodies conjugated to Alexa Fluor 488. No permeabilisation was involved. Coverslips were mounted on 5µl Mowiol placed on the slides and allowed to dry at RT. The slides were observed under the confocal microscope and the trails deposited by 300 parasites per coverslip in each condition were counted. With internal triplicates the experiment was performed twice. Mean

values  $\pm$  standard deviation (SD) from two independent biological experiments was calculated.

#### II-3.6 Structured Illumination Microscopy (SIM)

SIM was used to obtain high-resolution images using an ElyraPS1 microscope system (Zeiss) with a 100x oil-immersion lens (alpha Plan Apochromat 100x, NA 1.46, oil immersion) and a resolution of 120 nm along the x-y axis and 500 nm along the z-axis (PSF measured on 100 nm beads; Sampling voxel size:  $0,050\mu$ m\* $0,050\mu$ m\* $0,150\mu$ m). Three lasers (405, 488, and 561 nm) were used for excitation. SIM images were acquired with an EMCCD camera (Andor Technology Ltd, UK) and processed with ZEN software, where exposure times varied between 100 and 150 ms. Three-dimensional images were generated using a z-step of 150 nm (total thickness ~5  $\mu$ m). The acquisition was done sequentially using Zeiss Filter Sets 43HE, 38HE and BP 420-480. 15 frames were acquired to reconstruct one image (5 rotations x 3 phases, with a SIM Grating period of 51 $\mu$ m for the blue channel, 42  $\mu$ m for the green channel, 34 $\mu$ m for the red channel). 100 nm beads were imaged to measure the chromatic misalignment of our system (fit procedure by the Zen software); this parameter enabled correcting the alignment on each acquired multi-channel stack. Image reconstructions and co-localisation analysis were determined with IMARIS software (Bitplane).

#### II-3.7 Correlative light electron microscopy (CLEM)

Host cells were cultured on alphanumerical gridded-glass bottom dishes (ref **P35G-1.5-14-CGRD**, MatTek Corporation, Ashland, MA, USA) until 50% confluence was reached. Parasites were allowed to invade for 2 h, washed twice with warm medium, then induced for 6 h with Rapamycin 50nM, washed thrice with 1X PBS and allowed to grow for additional 16hrs. Cells were then fixed with 4% PFA / 0.5% glutaraldehyde in 1X PBS over-night. YFP-positive  $TgAP\mu$ 1-KO parasites were imaged using a Zeiss LSM880 confocal microscopy and localized on the alphanumerical grid using transmitted light. After observation, cells were fixed with 2% glutaraldehyde in 0.1 M sodium cacodylate buffer over-night. After washing with water, cells were sequentially stained with 1% osmium tetroxide reduced with 1.5% osmium tetroxide, 1% uranyl acetate overnight at 4°C, and finally lead aspartate for 3 h. All stains were made in water, in the dark and at room temperature unless otherwise indicated. All stains were also washed with water. After staining, cells were dehydrated in graded ethanol solutions, infiltrated with epoxy resin and cured at 60°C for 48 h. After separation of the resin from the glass, cells of interest were relocated with the imprinted-alphanumerical grid at the surface of the resin. Small blocks of resin containing the cells of interest were prepared for sectioning parallel to the resin surface. Serial sections of 80 nm thickness were set down on carbon/formvar-coated slot grids. Sections were observed with a Hitachi H7500 TEM (Elexience, France), and images were acquired with a 1 Mpixel digital camera from AMT (Elexience, France).

#### **II-3.8 Scanning Electron Microscopy (SEM)**

Freshly egressed Rab11A<sub>WT</sub> and Rab11A<sub>DN</sub> parasites were induced with 0.6 $\mu$ M and 1  $\mu$ M Shield-1 for 2 hours, respectively. 1\*10<sup>6</sup>parasites of each strain were seeded onto BSA (100 $\mu$ g/ml) coated coverslips for 30 minutes and then fixed with 2.5 % glutaraldehyde in 0.1M sodium cacodylate buffer for 5 hours. After washing, cells were treated with 1 % osmium tetroxide in water, in the dark, for 1 hour. Cells were dehydrated with increasing ethanol concentration baths. After two pure ethanol baths, cells were air-dried with HMDS. Finally dry coverslips were mounted on stubs and coated with 5 nm platinum (Quorum Technologies Q150T, Elexience, France). Cells were imaged at 2 kV by a secondary electron detector with a Zeiss Merlin Compact VP SEM (Zeiss, France).

#### **II-4.** Biochemistry

#### **II-4.1 Total protein extract and Western Blot:**

Parasites were lysed in lysis buffer (NaCl 150mM, TrisHCl 20mM, EDTA 1mM, 1% TritonX100, protease inhibitors) for 30 minutes at 4°C and the lysate was centrifuged for 15 min at 14000 rpm to remove cell debris. The lysate was then resuspended with 4X loading buffer (X) and total proteins were subjected to electrophoresis in a 10% or 12% polyacrylamide gel. The proteins were transferred onto a nitrocellulose membrane (Amersham<sup>TM</sup>Protran<sup>TM</sup> 0.45µm NC) by a standard western blot procedure. The membrane was blocked with 5% milk (non-fat milk powder dissolved in TNT buffer: 100mM Tris pH8.0, 150mM NaCl and 0.1% Tween20) and probed with primary antibodies diluted in the blocking buffer. The primary antibodies were followed by respective species specific secondary antibodies conjugated to HRP. The antibody incubations were followed by

thorough washing using the TNT buffer. The membranes were visualized using ECL Western blotting substrate (Pierce).

#### **II-4.2 Immunoprecipitation**

For immunoprecipitation assays, a minimum of 0.6 billion parasites were lysed on ice for 30 minutes in modified RIPA buffer (50mM TrisHCl pH8.0, 2mM EDTA, 75mM NaCl, 0.65% NP40, 0.005%SDS, 0.5mM PMSF) and centrifuged at 14 000 rpm for 15 min to remove cell debris. Protein concentration was determined using the BCA protein assay kit (Pierce<sup>TM</sup>). 500µg of total lysate were immunoprecipitated by binding to 50µl of anti-cmyc coated agarose beads (Pierce<sup>TM</sup>) or anti-HA agarose beads (Pierce<sup>TM</sup>) overnight. After five washes of 10 min each with modified RIPA buffer, bound proteins were eluted by boiling the samples in laemmeli buffer. Samples were then subjected to SDS PAGE and western blotting or gel-extracted for tryptic digestion and mass spectrometry analysis.

#### II-4.3 GST pull-down

The C-terminal ear appendage domain of the  $\beta$  (BAE) and  $\gamma$  (GAE) subunits, and full length Rab11A were GST tagged by cloning into a pGEX6p3 vector (Pharmacia) using the respective restriction sites mentioned in the table of primers listed above. Expression of GST-BAE, GST-GAE and GST-Rab11A in BL21 competent cells was achieved by induction with 1mM IPTG at 37°C for 4 h. Bacteria lysates expressing all GST recombinants and GST alone (control) were bound to 100µl of Protino Glutathione agarose 4B beads (Machery Nagel) in GST-lysis/binding buffer (Tris HCl (pH 7.6) 50mM, EDTA 1mM, EGTA1mM, 2mercaptoethanol 10mM, NaCl 150mM, TritonX-100 0.5%, and 0.5mM PMSF) overnight at 4°C. The beads were washed 5 times with wash buffer A (Tris HCl (pH 7.6) 50mM, 2mercaptoethanol 10mM, NaCl 500mM, Triton 0.5% and 0.5mM PMSF) and 3 times with wash buffer B (Tris HCl (pH 7.6) 20mM, NaCl 150mM, NP40 0.65%, SDS 0.005%, 0.5mM PMSF) sequentially. Beads containing 150µg of the recombinant proteins and the control GST protein were incubated with a lysate from 0.4 billion pLIC-EPSL-cmyc / pLIC-AP1µ1-HA or wildtype RHAKu80 intracellular parasites, overnight at 4°C. Parasites were lysed using modified RIPA (TrisHCl (pH8.0) 50mM, EDTA 2mM, NaCl 75mM, NP40 0.65%, SDS 0.005%, PMSF 0.5mM). After 3 washes with the lysis buffer, the proteins bound to the beads were eluted with 1x Laemelli blue buffer by boiling. The samples were subject to western blot and mass spectrometric analyses.

#### **II-4.4 Mass spectrometry and proteomic analysis**

After denaturation at 100°C in 5% SDS, 5% ßmercaptoethanol, 1 mM EDTA, 10% glycerol, 10 mM Tris buffer pH 8 for 3 min, protein samples were fractionated on a 10% acrylamide SDS-PAGE gel. The electrophoretic migration was stopped as soon as the protein sample entered 1 cm into the separating gel. The gel was briefly stained with Coomassie Blue, and five bands, containing the whole sample, was cut. In gel digestion of gel slices was performed as previously described (Miguet, L. et al., 2009). An UltiMate 3000 RSLCnano System (Thermo Fisher Scientific) was used for separation of the protein digests. Peptides were automatically fractionated onto a commercial C18 reversed phase column (75 µm×150 mm, 2 µm particle, PepMap100 RSLC column, Thermo Fisher Scientific, temperature 35 °C). Trapping was performed during 4 min at 5 µl/min, with solvent A (98 % H2O, 2% ACN and 0.1 % FA). Elution was performed using two solvents A (0,1 % FA in water) and B (0,1 % FA in ACN) at a flow rate of 300 nl/min. Gradient separation was 3 min at 5% B, 37 min from 5 % B to 30% B, 5 min to 80% B, and maintained for 5 min. The column was equilibrated for 10 min with 5% buffer B prior to the next sample analysis. The eluted peptides from the C18 column were analyzed by Q-Exactive instruments (Thermo Fisher Scientific). The electrospray voltage was 1.9 kV, and the capillary temperature was 275 °C. Full MS scans were acquired in the Orbitrap mass analyzer over m/z 300-1200 range with resolution 35,000 (m/z 200). The target value was 5.00E+05. Ten most intense peaks with charge state between 2 and 4 were fragmented in the HCD collision cell with normalized collision energy of 27%, and tandem mass spectrum was acquired in the Orbitrap mass analyzer with resolution 17,500 at m/z 200. The target value was 1.00E+05. The ion selection threshold was 5.0E+04 counts, and the maximum allowed ion accumulation times were 250 ms for full MS scans and 100 ms for tandem mass spectrum. Dynamic exclusion was set to 30s.

#### **II-5.5 Proteomic data analysis**

Raw data collected during nanoLC-MS/MS analyses were processed and converted into \*.mgf peak list format with Proteome Discoverer 1.4 (Thermo Fisher Scientific). MS/MS data was interpreted using search engine Mascot (version 2.4.0, Matrix Science, London, UK) installed on a local server. Searches were performed with a tolerance on mass measurement of 0.2 Da for precursor and 0.2 Da for fragment ions, against a composite targetdecoy database (50620 total entries) built with 3 strains of *Toxoplasma gondii* ToxoDB.org database (strains ME49, GT1 and VEG, release 12.0, September 2014, 25264 entries) fused with the sequences of

recombinant trypsin and a list of classical contaminants (46 entries). Cysteine carbamidomethylation, methionine oxidation, protein N-terminal acetylation and cysteine propionamidation were searched as variable modifications. Up to one trypsin missed cleavage was allowed.

#### **II-6.6 Antibody raising**

Recombinant purified GST-Rab11A protein was used to raise a Rab11A specific mouse polyclonal antibody. The Prescission site present between the GST tag and Rab11A was made use of by digesting the purified recombinant protein with Prescission protease (GE life science). The GST-Rab11A bound agarose beads were washed with 10 bed volume of Cleavage buffer (50mM Tris HCl, pH7.0, 150mM NaCl, 1mM EDTA, 1mM DTT) at 4°C. 20ul Prescission protease (40 units) was added to 980 ul cleavage buffer and incubated with the beads at 4°C overnight. The cleavage occurred on the beads and the GST was retained on the beads while purified Rab11A was collected in the supernatant. The purified protein suspended in Freund's adjuvant was injected intra-peritoneally into mice over a series of 4 boosts. Following the third boost a small sample of serum was collected the specific activity of the antibody in western blots and IFA, the mice were sacrificed at the end of the final boost and the serum was collected and stored at -20°C for future use.

#### **II-7.7 Statistics**

Means and SD or SEM were calculated in Excel. *P*-values were calculated in Excel using the Student's *t*-test assuming equal variance, unpaired samples and using two-tailed distribution.

### **III-RESULTS**

# **III-1.** Dual role for the *T. gondii* clathrin adaptor AP1 in the sorting of rhoptry and microneme proteins and in parasite division.

The heterotetrameric clathrin adaptor complex AP1 plays an important role in the secretory protein trafficking pathway. Previously work carried out in our lab on TgSORTLR (Sortilin Like Receptor) showed that this protein is the unique receptor loading ROP and MIC cargo proteins at the TGN and transports them to the endosomal-like compartments. Thus, TgSORTLR is essential for the biogenesis of the apical secretory organelles namely, the micronemes and rhoptries (Sloves, P.J. et al., 2012). Further it was demonstrated that while the lumenal domain of the protein interacts with all studied microneme and rhoptry proteins, the cytoplasmic tail recruits trafficking molecules involved in both the anterograde and retrograde pathways, among which, the clathrin adaptor complex 1 subunit  $\mu$ . By homology to what is known in other eukaryotes, we hypothesized that TgAP1 could participate in the differential sorting of microneme and rhoptry proteins from the TGN to their distinct target destinations. Our data indicate that TgAP1 localises mostly at the TGN and in numerous cytoplasmic vesicles. Co-immunoprecipitation experiments showed that the formation of a usual tetrameric complex is conserved in T. gondii and that the protein interacts with known partners, such as Arf1 and the unique Epsin protein of the parasite. Furthermore, using a Cre-Lox-based inducible Knock-Out strategy, we demonstrated that  $T_gAP1$  regulates the sorting and transport from the TGN of only a sub-population of microneme proteins, previously shown to depend on TgRab5A/C activity for their trafficking. Accordingly, TgAP1 defective parasites displayed morphological defects in the TgRab5A-positive endosome like compartment (ELC). In addition, our data suggest that TgAP1 is crucial for rhoptry biogenesis, regulating both, the exit of immature proteins from the TGN and the subsequent step of organelle maturation. Finally our study revealed a novel role for  $T_gAP1$  in the late stage of the cytokinesis process involved in daughter cell segregation.

This work has been communicated to PLOS Pathogens and is currently under revision. Here, we decided to present the article in its present state (not the original submitted version), which includes some of the experiments required by the reviewers. Therefore, some author

comments have been included in the text and the additional experiments that are currently performed are indicated at the end of each chapter in inserts. In particular, figure 6 of the present manuscript will be edited to a large extent and we are still investigating in detail the division defect observed in AP1-defective parasites. The final version of the manuscript will be submitted mid-December.

# Dual role of the *Toxoplasma gondii* clathrin adaptor AP1 in the differential sorting of rhoptry and microneme proteins and in parasite division.

Venugopal K.<sup>1</sup>, Werkmeister E.<sup>1</sup>, Barois N.<sup>1</sup>, Saliou J.M.<sup>1</sup>, Sindikubwabo F.<sup>2</sup>, Hakimi M.A.<sup>2</sup>, Langsley G.<sup>3</sup>, Lafont F.<sup>1</sup>, and Marion S<sup>1</sup>.

<sup>1</sup> Centre d'Infection et d'Immunité de Lille, Université de Lille, CNRS UMR 8204, Inserm U1019, CHU Lille, Institut Pasteur de Lille, F-59019 Lille, France

<sup>2</sup> IAB, Team Host-pathogen interactions & immunity to infection, Université Grenoble Alpes, INSERM U1209, CNRS UMR5309, 38700 Grenoble, France

<sup>3</sup> Laboratoire de Biologie Cellulaire Comparative des Apicomplexes, Faculté de Médicine, Université Paris Descartes - Sorbonne Paris Cité, France. Inserm U1016, CNRS UMR8104, Cochin Institute, Paris, 75014 France.

#### Abstract

*Toxoplasma gondii* possesses a highly polarized secretory system, which efficiently assembles *de novo* micronemes and rhoptries during parasite replication. These apical secretory organelles release their contents into host cells promoting parasite invasion and survival. Using a CreLox-based inducible knock-out strategy and the ddFKBP over-expression system, we unraveled novel functions of the clathrin adaptor complex *Tg*AP1 in the sorting of a specific sub-population of microneme proteins and in the regulation of rhoptry biogenesis. In particular, our data indicate a role for *Tg*AP1 in modulating early endosomal compartment morphology during the anterograde transport of ROP proteins towards the apical pole of the parasite. In addition, we identified *Tg*EpsL (Epsin-like protein), the unique *T. gondii* ENTH-domain containing protein, as a key partner of *Tg*AP1. Similar to other eukaryotes, *Tg*EpsL binds to *Tg*AP1 in the regulation of parasite division during the late stage of daughter cell segregation.

#### **Author Summary**

The phylum Apicomplexa comprises a large group of obligate intracellular protozoan parasites of world-wide human and agricultural significance. Most notable is *Plasmodium*, the causative agent of malaria, and *Toxoplasma gondii*, one of the most common human parasites, responsible for Toxoplasmosis, an opportunistic disease that affects the developing fetus during primo-infection of the pregnant woman and immune compromised individuals. Apicomplexa are characterized by the presence of an apical complex consisting of secretory organelles named micronemes (MIC) and rhoptries (ROP). MIC and ROP proteins, released upon host cell recognition, are essential for host cell invasion and parasite survival. After invasion, these organelles are neo-synthetised at each parasite replication cycle. In our study, we demonstrated a crucial role for the *T. gondii* clathrin adaptor complex AP1 in the vesicular transport of neo-synthesized microneme and rhoptry proteins, thereby regulating mature apical organelle formation. In addition, we unraveled an original role for *Tg*AP1 in the division process of the parasite. Therefore, our study provides new insights into crucial regulatory mechanisms of the vesicular trafficking system essential for host invasion and intracellular survival of *Toxoplasma gondii*.

#### Introduction

Eukaryotic parasitic pathogens belonging to the phylum *Apicomplexa* are responsible for causing severe mortality in humans and great economic losses in livestock. *Toxoplasma gondii* (*T. gondii*) is of critical importance to pregnant women, as primary infections have the potential to cause neonatal malformations and even death of the developing foetus. In addition, the opportunistic nature of this obligate intracellular parasite can lead to the development of encephalitis in immunocompromised individuals after reactivation of lifelong persistent cysts in the central nervous system [1]. As its name suggests *Apicomplexa* have a complex of unique apical secretory organelles called the micronemes, rhoptries and dense granules that sequentially release their contents into the host cytoplasm enabling parasite invasion and intracellular survival. Microneme-specific MIC proteins are first secreted and trigger the formation of a transient structure, the moving junction (MJ) [2] [3], which promotes parasite penetration into the host cell. Rhoptry-specific ROP proteins are immediately

discharged after the MIC proteins and participate in the formation of the MJ and establishment of the intracellular parasitophorous vacuole (PV) in which the parasite intensively multiplies. ROP proteins secreted into the host cell also play a crucial role in the manipulation of host innate immune responses to promote parasite survival [4]. Dense granule proteins (GRAs) are key parasite effectors exocytosed during parasite entry into the vacuolar space, where a certain sub-population contributes to the formation of a nano-tubulo-vesicular network called the intravacuolar network [5] [6]. This tubular network has been shown to be essential for nutrient import and regulation of parasite antigen exposure at the PV [7]. GRA proteins are also inserted into the PV membrane and previous studies have suggested that they may trigger recruitment of host cell organelles, such as the Golgi apparatus and endoplasmic reticulum (ER) [8][9]. In addition, similar to ROP proteins, GRA proteins can be secreted beyond the PV membrane to actively modulate host gene expression and immune responses that are triggered upon infection [10].

The stripped-down and polarized version of the eukaryotic intracellular trafficking system has facilitated the use of *T. gondii* in studying the biogenesis of conserved organelles like the Golgi apparatus [11], and more recently the rhoptries and micronemes [12]. Rhoptries, micronemes and dense granules are formed *de novo* during each parasite replication cycle by budding and fusion of vesicles emerging from the ER and Golgi. Earlier studies have characterized the sorting motifs within MIC and ROP proteins required for their trafficking from the Golgi towards their final destination [13][14][15][16]. These studies led to the conclusion that protein processing and protein sorting were inter-dependent activities. More recently the trafficking routes taken by MIC and ROP proteins were delineated by examining the functions of some regulators of the endocytic compartments [12] [17]. Key trafficking molecules were identified, such as the sortilin-like receptor (SORTLR) [18], the dynamin-related protein B (DrpB) [19] and the HOPS/CORVET complex protein Vps11 [20], all involved in the anterograde pathway regulating secretory organelle biogenesis. In addition, TgStx6, a parasite SNARE homolog of syntaxin 6 and the retromer protein Vps35, which are involved in retrograde transport of molecules from the endosomal compartment to the Golgi, were shown to be

required for the biogenesis of dense granules [21] and rhoptries/micronemes [22], respectively. These recent findings have suggested that T. gondii has functionally repurposed evolutionarily conserved regulators of the endosomal system to the secretory pathway to form secretory organelles [12]. TgSORTLR was identified as the unique receptor transporting both, ROP and MIC proteins from the Golgi to the endosomal-like compartment [17]. Depletion of TqSORTLR led to parasites deprived of apical secretory organelles such that ROP and MIC proteins were released into the vacuolar space, or the host cell cytoplasm via the default constitutive secretion pathway. So far, little is known about the molecular machinery regulating the differential sorting of ROP, MIC and GRA proteins from the ER/Golgi towards their distinct final destinations. ROP proteins and a subpopulation of MIC proteins (MIC3/MIC8 complex) were shown to be transported via a TgSORTLR- and TqRab5A/C-dependent but Rab7-independent pathway, while MIC2/M2AP complex trafficking was TqSORTLR-dependent, but TqRab5A/C- and TgRab7-independent [17] [18]. However, depletion of Mon1, the putative GEF factor for Rab7, was recently shown to impair ROP, MIC and GRA formation, leading to contradictory conclusions concerning the role of Rab7 in secretory organelle formation [20]. Interestingly, the clathrin adaptor protein 1 (TgAP1) complex was found associated with the Cterminal tail of TgSORTLR, suggesting that TgSORTLR-mediated transport of ROP and MIC proteins occurs via TgAP1- and clathrin-dependent budding from the Golgi [17]. In eukaryotes, the AP1 complex has a highly conserved regulatory function in the bidirectional transport of proteins between the Trans-Golgi-Network (TGN) and the early / sorting endosomal compartment [23]. AP1 has been also shown to have a conserved role in the regulation of the cell division process in lower and higher eukaryotes. AP1 is crucial for the final step of daughter cell segregation by delivering Golgi-derived vesicles at the cleavage furrow of dividing cells. AP1 is composed of 4 sub-units: two large sub-units  $\gamma$  and  $\beta$ , a medium sub-unit  $\mu$ , and a small sub-unit  $\sigma$ . Sorting motifs present in the cytoplasmic domain of cargo receptors are specifically recognised by the AP1 complex [23] [24]. In T. gondii, ROP2 possesses a diLeucine and a Tyrosine-based motif, both required for export of ROP proteins from the TGN to form mature organelles [25]. The Tyrosine-based motif of membraneassociated ROP2 is specifically recognized by the µ sub-unit of *Tg*AP1 [26]. Over-expressing mutated *Tg*APµ1 (D176A), or the Tyrosine-motif mutated in ROP2 led to accumulation of ROP2 in a post-Golgi multi-vesicular compartment resembling endosomes and immature rhoptries [26]. In addition, perturbing the function of *Tg*APµ1 by siRNA interference led to major defects in ROP biogenesis while, microneme and dense granule organelle biognesis was not perturbed [26]. *Tg*SORTLR that associates with ROP and MIC proteins also possesses a diLeucine motif in its cytoplasmic tail suggesting an additional *Tg*AP1-dependent sorting mechanism for soluble ROP and MIC proteins. In *P. falciparum*, AP1 localises at the Golgi/ER and rhoptries in schizont stages. The APµ1 sub-unit was found associated with the rhoptry associated protein 1 (RAP1) suggesting a role in rhoptry protein trafficking.

Here, using a CreLox-based inducible knock-out strategy, we demonstrated that *Tg*AP1 regulates rhoptry and microneme formation. However, our data indicated that AP1 regulates the sorting and transport of only a sub-population of MIC proteins from the TGN previously shown to be dependent on TgRab5A/C activity. In agreement, *Tg*AP1-overexpressing parasites accumulated enlarged vacuolar *Tg*Rab5A-positive endosomes retaining pro-ROP proteins. In addition, our study revealed a novel role for *Tg*AP1 in late stages of cytokinesis, presumably by regulating key events required for final daughter cell abscission. Finally, we identified the unique *T. gondii* ENTH-domain containing protein (named *Tg*EpsL, for Epsin-Like protein) as a preferential partner of *Tg*AP1 and depletion of *Tg*EpsL also led to defects in apical secretory organelle formation.

#### RESULTS

#### TgAPµ1 localizes at the Trans-Golgi-Network and on secretory vesicles

In order to define the localisation of the TgAP1 complex, we generated knock-in (KI) parasites expressing the µ1 sub-unit (TGGT1\_289770) fused to a HA tag at its C-terminus. Western blot analysis confirmed the expression of the tagged protein at the expected size of 49kDa (Fig 1A). A clonal parasite line was isolated and TgAPµ1 localisation was ascertained by an immunofluorescence assay (IFA) using confocal (Fig 1B) and super-resolution microscopy SIM (Structured Illumination Microscopy) (Fig 1C and 1D). As expected for the AP1 complex, a localisation at the Golgi area was observed and confirmed by co-localisation with the Trans-Golgi-Network (TGN) marker *Tg*SORTLR (Fig 1B, 1C). In addition to the TGN, a faint but specific *Tg*APµ1 signal was systematically detected in the parasite cytoplasm by confocal microscopy (Fig 1D). After saturating the higher Golgi associated signal, we clearly identified this weaker signal as *Tg*APµ1-positive vesicles spread throughout the cell cytoplasm and close to the cell periphery (Fig 1D), suggesting an additional role of the AP1 complex in secretion to the plasma membrane.

SIM acquisition and Imaris software analysis indicated 46,4  $\pm$  3,8% co-localisation between *Tg*APµ1 and *Tg*SORTLR (Fig 1C and 1F) at the TGN. In agreement, co-localisation analysis by SIM between *Tg*APµ1 with another TGN markers, GalNAc, shows a similar high percentage of co-localization (52 $\pm$  5,6%) (Fig 1E and 1F) in contrast to the cis-Golgi marker GRASP (Fig 1E and 1F). Using a knock-in line expressing Rab5A-YFP under the natural promotor, the Rab5A-positive endosome-like compartment (ELC) was mostly detected as vesicles emerging posteriorly from the *Tg*APµ1- and *Tg*SORTLR-positive TGN (Fig 1E and movies SM1 and SM2). In agreement, a weaker co-localization of *Tg*APµ1 with *Tg*Rab5A was monitored (22,9  $\pm$ 8,2 %) compared to the TGN markers TgSORTLR and GalNac (Fig 1E and 1F). However, we noticed that the Rab5A-positive ELC and the TGN appeared physically connected, in particular during Golgi duplication at the G1/S phase of the cell cycle (Fig S1A). This notion was supported by the observation that BFA treatment led to partial dispersion of the *Tg*Rab5A-positive and TgRab7-positive compartments similar to what was observed for *Tg*APµ1 (Fig S1B). This result indicates that *T. gondii* possesses an unusal endosome-like compartment, physically endosome / TGN hybrid compartment of plants.

*T. gondii* APµ1 belongs to a conserved tetrameric complex and interacts with the epsin-like protein To assess whether TgAPµ1 is a component of the highly conserved heterotetrameric complex composed of the 3 other sub-units:  $\sigma 1$ ,  $\beta 1$  and  $\gamma 1$ , we performed an immunoprecipitation assay (IP) using anti-HA antibodies on TgAPµ1-HA knock-in (KI) parasite lysate followed by mass spectrometry
analysis. TgAPµ1 was reproducibly found associated with the other 3 sub-units (Table I) confirming the formation of a conventional heterotetrameric complex described for other eukaryotes. This finding was also supported by the localisation of the  $\sigma 1$  sub-unit at the TGN in KI parasites expressing the fusion protein  $\sigma$ 1-HA under the native promotor (Fig S2A, S2B). In addition, the unique ENTH domain-containing protein encoded in the T. gondii genome, which we named Epsin-like protein (TqEpsL) was identified, however with only one unique peptide (Table I). We investigated further the interaction between TqAP1 and TqEpsL because epsin proteins are very well known AP2 and AP1 complex binding proteins involved in the activation of clathrin-mediated vesicular budding at the plasma membrane and at the TGN, respectively. Epsins bind to phospholipids via their ENTH domain and regulate clathrin coat formation by inducing curvature of the lipid bilayers [24]. Sequence analysis indicated that TgEpsL contains conserved clathrin and phosphoinositide binding sites, such as recently described elsewhere [27] (Fig S2C). However, besides the ENTH domain, no similarities were found between all studied epsins, suggesting a specificity of binding partners and regulatory mechanisms of the protein activity. First, we generated double KI parasites expressing both, TgEpsLcMyc and  $TqAP\mu 1$ -HA proteins under their natural promotors (Fig 2A). IFA analysis confirmed the colocalisation of TgAP1 and TgEpsL at the TGN, together with TgSORTLR (Fig 2B). To verify the interaction between TqAP1 and TqEpsL, an IP was performed in double KI TqEpsL-cMyc / TqAP $\mu$ 1-HA parasites, using either anti-cMyc or anti-HA antibodies. Western blot analysis confirmed the interaction between TqAPµ1-HA and TqEpsL-cMyc in both IP assays (Fig 2C and 2D). In agreement, IP of TgEpsL-cMyc followed by mass spectrometry identified the  $\beta$ ,  $\gamma 1$  and  $\mu 1$  sub-units of the AP1 complex and the small GTPase ARF1 as the main proteins associated with TqEpsL (Table II), confirming the result obtained by western blot. Importantly, no sub-unit of the AP2 complex was identified, suggesting that TqEpsL might not function in AP2-mediated endocytosis. In other eukaryotes, epsinR interacts with the AP1 complex preferentially via the exposed GAE ("Gamma Appendage Ear") domain of the  $\gamma$  sub-unit [28] [29]. Sequence alignment analysis showed a strong conservation of T. gondii GAE and BAE ("Gamma Appendage Ear" and "Beta Appendage Ear") domains with domains contained in the AP1  $\beta$  and  $\gamma$  sub-units from other species (Fig S3). Therefore, the ear appendage domain of the  $\beta$  and  $\gamma$  sub-units of *Tg*AP1 fused to GST were produced (Fig 2E, 2F). GST pull-down experiments in presence of a total extract of double *Tg*EpsL-cMyc/*Tg*µ1-HA KI parasites indicated that the ear domain of the  $\gamma$  sub-unit is sufficient to pull-down the *Tg*EpsL protein, while no-binding of the *Tg*APµ1 sub-unit was detected (Fig 2F). In contrast, a weak interaction was detected between *Tg*EpsL and the  $\beta$ -ear domain, showing the preferential association of *Tg*EpsL with the  $\gamma$ -ear (Fig 2F). Interestingly, the  $\beta$ -ear interacted with *Tg*µ1. As no direct interaction between this domain and the µ1 sub-unit has been described in other eukaryotes, it is likely that the  $\beta$ -ear pulls-down a complex of proteins that includes µ1 and other *Tg*AP1 binding proteins. In agreement, *Tg*SORTLR that directly interacts with the µ1 sub-unit, was also found in the pull down eluate of the  $\beta$ -ear but not the  $\gamma$ -ear (Fig 2F).

Together these data indicate that the AP1 complex in *T. gondii* is conserved at the molecular level and likely functions as its mammalian homologue as a heretotetrameric complex regulating clathrinand epsin-mediated vesicular transport of parasite proteins from the TGN/ELC compartment.

#### TgAP1 regulates the biogenesis of a sub-population of micronemes

To investigate *Tg*AP1 functions, inducible knock-out (KO) parasites lacking the  $\mu$ 1 sub-unit were generated using the CreLox strategy [30], where excision of the endogenous locus and subsequent expression of the YFP protein is triggered upon addition of rapamycin (Fig 3A). Integration of the LoxP- $\mu$ 1HA-LoxP cassette at the endogenous locus was validated by PCR (Fig 3B) and expression of the corresponding protein by Western Blot (Fig 3C). By IFA, expression of integrated  $\mu$ 1-HA gene, led to a tagged protein that co-localised with *Tg*SORTLR at the TGN (Fig 3D). Incubation of transgenic parasites with rapamycin triggered *Tg*AP $\mu$ 1 depletion and YFP expression, but only in 12 ±4 % of the whole population (Fig 3D). Of note, increasing rapamycin concentration did not lead to a higher yield of KO parasites. Longer rapamycin induction periods improved the rate of YFP-positive parasites but led to ramdom excision events over the successive cell cycles rending difficult the analysis of the phenotypes. We thus decided to induce AP $\mu$ 1-KO parasites 4 hours post-invasion in order to trigger  $\mu$ 1 gene exision during the first division cyle.

TqAPµ1 had been previously identified associated with the C-terminal tail of TqSORTLR, suggesting TqAP1-mediated transport and sorting of microneme and rhoptry proteins during organelle biogenesis [18]. We therefore investigated the formation of the two distinct microneme populations previously described [17]: the MIC3/MIC8 complex transported via a TgSORTLR /TgRab5-dependent pathway and the M2AP/MIC2 complex via a TgSORTLR-dependent/TgRab5-independent pathway. Upon depletion of TgAPµ1, soluble protein MIC3 was re-directed towards the vacuolar space, demonstrated by the GAP45 labeling of parasite contours, whereas MIC8 was found retained in the Golgi, indicated by its co-localisation with TgSORTLR (Fig 4). A staining for MIC8 at the plasma membrane was also detected suggesting that a part of the protein escaped by the constitutive secretory pathway to the parasite surface (Fig 4). On the other hand, M2AP seemed to be correctly targeted to micronemes, however, MIC2 appeared concentrated at the apex of the parasites, while lateral micronemes were weakly detected (Fig 4). In agreement to the hypothesis of TgAP1 being only involved in MIC3/MIC8 traficking pathway,  $TqAP\mu1$  co-localised with immature pro-MIC3 but not with immature pro-M2AP (Fig S4A). Of note, TgSORTLR was not mis-localised in TgAPµ1-KO parasites (Fig S4B), suggesting that TgAP1 functions downstream of TgSORTLR in the anterograde secretory pathway. Finally, we observed that dense granule biogenesis was not affected in APµ1-KO parasites (Fig S4C). Therefore, our data indicate that TgAP1 is part of the early sorting machinery that differentially directs the MIC proteins to distinct destinations from the TGN, and seems functionally correlated to the TgRab5A/C compartment, the two regulating similar trafficking pathways for MIC biogenesis [17].

#### AP1 regulates rhoptry biogenesis

In YFP-positive parasites, deletion of  $Tg\mu 1$  drastically affected the formation of rhoptry organelles, which appeared as fragmented compartments distributed throughout the cell cytoplasm (Fig 5A,

upper panel and Fig 5B) or in the vacuolar space (Fig 5A, middle panel). These ROP 2-4 containing fragmented compartments did not correspond to TaSORTLR-positive TGN-associated (Fig 5A) or immature ProROP4-positive compartments (not shown). Of note, it has been previously shown that while de novo rhoptry formation takes place in nascent daughter cells, mother rhoptries are actively degraded. To address this aspect, we monitored immature pre-rhoptry compartment formation in TqAPµ1-KO parasites (Fig 5A and 5C). Quantification of parasites displaying a positive signal for Pro-ROP4 proteins indicated that 26,1 ±1,2 % of control parasites contained immature rhoptries, corresponding to dividing parasites in M phase of the cell cycle. In TgAPµ1-KO parasites, an increase in the percentage of vacuoles positive for the pro-ROP4 signal was counted (40,1 ±1,3%), indicating no defect in ROP protein neosynthesis. However, the pro-ROP4 proteins were detected in the vacuolar space or the residual body in 72  $\pm$ 4.4% of the ProROP4-positive vacuoles (Fig 5A and 5C). These data suggest that the TgAP1 complex is involved in immature pro-ROP protein exit from the TGN/ELC compartments. In absence of AP1, these proteins are likely targeted to the constitutive pathway towards the vacuolar space. The fragmented ROP2-4 positive compartments that we detected in APµ1-KO parasites might correspond to remnant mother cell rhoptries undergoing degradation but this observation could also suggest an additional role of AP1 in the later stages of rhoptry maturation at a post-Golgi level.

Finally, in agreement to the defect in rhoptry and microneme formation, we also found that host cell invasion was perturbed in  $TgAP\mu1$ -KO parasites (Fig 4D).

#### **Fig 5**. *Tg***APµ1 knock-out impairs rhoptry formation**. Egress activity is under quantification in AP1-KO parasites

#### Over-expression of AP $\mu$ 1 led to defects only in rhoptry formation

It has been previously shown that over-expression of wildtype (WT)  $TgAP\mu 1$  or a point-mutated dominant negative mutant of  $TgAP\mu 1$  caused a drastic defect in rhoptry formation at the post-Golgi level, while microneme biogenesis was not impaired [26]. To understand the distinct phenotype

observed compared to the  $TgAP\mu1$ -KO parasites, WT  $Tg\mu1$  sub-unit was conditionally over-expressed (DDcMycu1 parasites) using the ddFKBP system [31]. Western blot (WB) analysis confirmed the timedependent accumulation of the cMycµ1 sub-unit upon shield-1 induction (Fig 6A). The overexpressed DDcMycµ1 protein partially localised in the Golgi area but also in cytoplasmic vesicles particularly concentrated at the basal pole of dividing parasites (Fig 6B). We found that DDcMycµ1overexpressing parasites displayed much milder defects compared to APµ1-KO parasites, which were similar to what has been previously described in [26]. ROP proteins were mostly detected in vacuolar or atrophied/fragmented compartments (Fig 6C). However, in DDcMycµ1 induced parasites, Pro-ROP4 proteins were not detected in the vacuolar space or in the residual body, as observed in APu1-KO parasites, but co-localised with the ELC marker TgRab5A (Fig 6D), suggesting a defect in a distinct trafficking step of the secretory pathway. In contrast to ROP proteins, MIC2/M2AP and MIC8/MIC3 complexes were correctly targeted to micronemes as previously observed after over-expression of the mutated  $Tg\mu 1D176A$  sub-unit [26] (Fig 6C, bottom). These observations obtained by fluorescence microscopy were confirmed using electron microscopy analysis (Fig S5A). While micronemes were still detected at the apex of the parasite, rhoptries were no longer visible and parasites accumulated large lucent vacuoles (V) (Figure S5A). Importantly, parasite morphology was also affected, together with parasite organisation within the vacuole, suggesting additional roles for TgAP1 in the maintenance of parasite cortical integrity. This defect might be linked to a TgAP1-dependent secretory activity at the plasma membrane as suggested by the presence of  $T_{g}$ AP1-positive vesicles at the parasite periphery (Figure 1D). Furthermore, in agreement with the observed defect in mature rhoptry formation, a partial decrease in host cell invasion was monitored (Fig 6E). Importantly,  $TqAP\sigma$ 1-HA correctly localised at the TGN, suggesting that DDcMycµ1 over-expression does not cause the mis-location of the endogenous TgAP1 complex (Suppl Fig 5B). In addition, immunoprecipitation of DDcMycµ1 using anti-cMyc antibodies identified the three other sub-units of the TqAP1 complex and TqEpsL as the main DDcMycµ1 protein partners (Table III). Together, these data indicate that the over-expressed cMycµ1 protein integrates into the endogenous AP1 complex,

suggesting that the defect in ROP biogenesis is not due to an indirect effect linked to the titration of an APµ1 binding partner. Therefore, our data indicate that over-expressed DDcMycµ1 protein solely impaired rhoptry maturation presumably by perturbing a sub-population of *Tg*AP1 complexes whose activity is only involved in post-Golgi trafficking functions and does not perturb MIC and immature Pro-ROP protein exit from the TGN.

#### Fig 6. The inducible over-expression of $TgAP\mu 1$ only perturbed rhoptry formation.

- Quantification of the % of vacuoles showing normal mature Rhoptry formation
- Quantification of the % of vacuole showing immature pre-ROP compartment formation
- Co-localisation analysis of ROP and pro-ROP proteins with the TGN (SORTLR) and ELC (Rab5A) compartments
- Egress activity of DDcMycµ1 expressing parasites.
- ROP and MIC protein processing by western blot

#### TgAP1 regulates TgRab5A compartment morphology

As DDcMycµ1 over-expressing parasites did not exhibit Pro-ROP proteins escape into the vacuolar space, but rather a post-Golgi defect in rhoptry maturation, we investigated further at which step of the secretory pathway rhoptry biogenesis was impaired in these parasites. *Tg*AP1 distribution in relation to the ELC (*Tg*Rab5A) was examined in more detail during the rhoptry maturation process by SIM microscopy (Fig 6). In control parasites, at the onset of ROP protein neo-synthesis during parasite division, pre-ROP compartments seemed to emerge from the TGN as already formed large vesicular compartments co-distributing with *Tg*Rab5A-positive vesicles or displaying a faint Rab5A signal at their limiting membrane (Fig 7A, top and Fig 7B). More distant pre-ROP compartments en route to the apical pole of the parasites, were detected as *Tg*Rab5A-negative compartments (Fig 6B, top, arrows). In DDcMycµ1 over-expressing parasites, pro-ROP4 proteins emerging from the Golgi were found mostly retained in larger compartments displaying an intense *Tg*Rab5A signal at their limiting membrane (63,6 ± 18,2 % compared to 21,9 ± 13 % for control parasites) (Fig 7A, 7B, bottom and 7C). The formation of *Tg*Rab5A-positive enlarged compartments empty of pro-ROP proteins could be also observed (Fig 7B, bottom). As these were never observed in control parasites, it argues

for a more general process of *Tg*AP1-dependent regulation of early endosomal membrane dynamics, such as already described in mammalian cells.

Therefore, the study of the milder phenotype associated with over-expression of the WT APµ1 subunit allowed us to identify an additional later step of AP1-mediated regulation of ROP trafficking at the level of the endosome-like compartment during the rhoptry maturation process.

Collectively, these data indicate that TgAP1 acts at different trafficking steps during secretory organelle biogenesis. First, TgAP1 regulates the anterograde transport from the TGN of only a subpopulation of microneme proteins, MIC3 and MIC8, which has been shown to depend on TgRab5A/C, but not MIC2 and M2AP. The soluble MIC3 protein escaped via the constitutive secretory pathway to the vacuolar space, while the transmembranes MIC8 was retained at the Golgi level or escaped towards the plasma membrane. Second, our data suggest that  $TgAP\mu1$  regulates rhoptry formation by acting at the level of immature ROP protein exit from the TGN/ELC as well as at a post-Golgi level during the rhoptry maturation process.

**Fig 7.** *Tg*AP1 regulates *Tg*Rab5A compartment dynamics and rhoptry maturation at a post-Golgi level - Co-localisation between Rab7 and Pro-ROP4 in DDCMycμ1 parasites

#### AP1 regulates cell division

When observed by IFA, both DDcMycµ1-overexpressing and the *Tg*APµ1-KO transgenic parasites display a clear cell division defect. Cell growth quantification indicated aberrant division from stage 8 to 16 and 4 to 8 parasites per vacuole in DDcMycµ1 parasites and APµ1-KO, respectively (Fig 8A, 8B). In both transgenic lines, division evolves towards the formation of disorganised vacuoles containing an uneven number of parasites, unlike the typical rosette-like structures observed in control parasites (Fig 8B). In APµ1-KO parasites, we found that centrosomes and centromeres replicate normally (Fig 8C) suggesting a defect in the cell cyle after the G1/S phase. To investigate this aspect, we took advantage of the dynamic localisation of the centromeric protein chromo1 during the different stages of the cell cycle (Fig 8C). As previously described (Gissot et al., 2012), chromo1 is detected as 1 dot (1 Centromere-Chromo1-positive/1 Nucleus: 1CC/1N) in G1 phase, as two dots in S

phase when centromeres duplicate (2CC/1N), 2 dots (2CC/2N) in late M phase when the two daughter nuclei separate by the newly formed daughter IMCs and finally chromo1 is no longer detected at the centromers (OCC/2N) but sometimes detected at the basal pole at the end of cytokinesis when the two daughter parasites segregate (Figure 8C). Compared to control parasites, APµ1-KO parasites displayed a higher percentage of parasites in M phase compared to G1/S phase, in particular in the late stage of cytokinesis (OCC/2N), suggesting a defect in daughter cell segregation rather than in DNA / centrosome duplication and in the budding process (Fig 8D). To confirm these data, we examined the budding process in APµ1-KO by monitoring IMC formation. We found that APµ1-KO displayed a similar percentage of IMC3-positive budding daughter cells compared to control parasites (Fig 8E and 8F). Notably, in these parasites, the formation of daughter IMCs appeared to be completed (Fig 8E, IMC1 labeling). Next, we examined the recruitment of the basal complex MORN1, which was shown to be required for the final step of daughter cell segregation (46). We detected MORN1 recruitment at the basal pole of APµ1-KO parasites, however, parasites often seemed to remain attached in a disorganised manner by the basal pole, where MORN1 displays a strong signal and enlarged structure compared to the sharp ring-like structure observed in control parasites (Fig 8 G). Finally, Correlative Light Electron Microscopy (CLEM) was used to examine in detail the morphological defects in TqAPµ1-KO parasites. Following rapamycin induction, YFP-positive parasites were spotted on an alphanumerical gridded coverslip and processed for EM analysis (Fig S4). TgAPµ1-KO parasites compared to neighbouring YFP negative parasites (Fig 9 A-B) were characterized by gross morphological defects associated with the cortex integrity (Fig 9 C-D). In less affected vacuoles, distant parasites seem to be bound by their basal body via membranous structures (Fig 9E). Apical micronemes could be observed (Fig 9F), however, no mature rhoptries were detected but we often observed large vesicular lucent compartments such as observed by confocal microscopy and TEM in DDcMycµ1 parasites (Fig 9 F-G).

# We are currently investigating MORN1 dynamics by live imaging after having obtained a clonal population of loxPAPµ1 parasites expressing MORN1-cherry. Our goal is to investigate whether AP1

would play a role in the dynamics of the basal complex by the local delivery of regulating factors that would be required to terminate daughter cell segregation. An alternative hypothesis that we are also investigating is the putative role of AP1 in the delivery of de novo synthesized lipids required to separate the newly formed daughter cell plasma membranes.

Fig 8. TgAP1 regulates the parasite division process at a late stage of cytokinesis.

- MORN1-Cherry live imaging in APµ1-KO parasites

- Improved Correlative Light Electron Microscopy (CLEM) to detect defects in plasma membrane segregation

- localization of membrane proteins such as the glucose transporter GT1 in AP1-KO parasites

#### Role of EpsL

We are currently generating a stable KO line of TgEpsL using the CRISPR-Cas 9 approach as well as the Tet-inducible system to obtain an inducible knock-down line for TgEpsL. Our goal is to analyse the role of TgEpsL in rhoptry and microneme biogenesis.

#### DISCUSSION

#### Role of *Tg*AP1 in rhoptry and microneme protein trafficking.

In this study, we found that *Tg*AP1 regulates the sorting of only a sub-population of microneme proteins, previously shown to traffic via a *Tg*Rab5-dependent pathway [17]. In *Tg*APµ1 depleted parasites, soluble MIC3 was re-directed towards the vacuolar space via the constitutive secretory pathway, whereas the transmembrane MIC8 was retained in the Golgi. This indicates a role for *Tg*AP1 in the anterograde pathway for MIC protein export from the TGN. In contrast, perturbing *Tg*AP1 functions resulted in ROP proteins being retained in *Tg*Rab5A-positive enlarged compartments and the formation of aberrant mature rhoptries. Notably, pro-ROP proteins were not rerouted into the vacuolar space or into the host cytosol as observed for the *Tg*SORTLR-KO parasites. Rhoptries and pre-rhoptries are acidified organelles packaged with specialized hydrolases and intra-luminal membranes therefore resembling multi-vesicular bodies (MVB) of mammalian cells [33]. The

mechanism of pre-ROP and mature rhoptry formation is unknown but might share similarities with the mechanism involved in the formation of MVB in plants, in particular due to the repositioning of the early endosomal compartment at the TGN in both organisms [34]. In plants, MVB or PVC (prevacuolar compartments) were shown to directly emerge from the hydrid TGN/EE as immature compartments containing intra-luminal membranes [35]. Furthermore, AP1 was shown to be critical for BFA-sensitive but wortmannin-insensitive post-Golgi trafficking events from the TGN/EE to the MVB [36][37]. In addition, fully differentiated APµ1-KO plant cells contained fragmented vacuoles rather than a large central vacuole as in wild type, suggesting an additional role of AP1 in vacuolar fusion similar to what is observed in yeast [28]. Our SIM microscopy images suggest that similar mechanisms might exist between plants and T. gondii for the formation of rhoptries. Pre-ROP compartments emerged from the TGN as vacuoles closely associated with the TgRab5 compartment. In addition, perturbing TgAP1 function resulted in a drastic increase in pre-ROP compartments strongly positive for TgRab5 at their limiting membrane, as well as an accumulation of fragmented rhoptries. Therefore, AP1 in T. gondii may well be involved in both steps of pro-ROP protein exit from the TGN/ELC compartment but also at the later step of pre-ROP vacuolar compartment maturation into club-shaped rhoptries. In mammalian cells, AP1 regulates retrograde transport of proteins from the early/sorting endosomal compartment back to the TGN, while GGA (Golgi-localized, y earcontaining, ADP-ribosylation factor-binding) is involved in anterograde transport from the TGN to the endosomes [23]. GGA molecules are not encoded in the T. gondii genome [12]. A blockade of both, anterograde and retrograde transport of proteins from the Rab5 endosomes would lead to accumulation of membrane and expansion of these compartments. Presently, we cannot fully exclude a similar role for TqAP1 in the retrograde transport of molecules from the ELC to the TGN. However, in parasites defective for TgAP1 we did not find an accumulation of molecules such as the ROP and MIC receptor TgSORTLR in TgRab5A/pre-ROP expanded compartments (not shown). Moreover, perturbing TgAP1 function induces a more general defect in the TgRab5A-positive compartment that expanded to become larger vacuolar compartments, distinct from immature pre-

ROPs. Therefore, taken together, the data argue for a specific functional relationship between *Tg*AP1 and the *Tg*Rab5A-positive compartment in *T. gondii*. In mammalian cells, AP1 and GGA1 proteins have been demonstrated to regulate Rab5 membrane dynamics by binding directly to the Rab5 effector Rabaptin5 [38] [39]. Notably, over-expression of Rabaptin 5 shifts the localization of GGA1 and TGN associated cargos into enlarged Rab5 endosomes [39] [40]. Therefore, further studies are needed to explore a putative role of *Tg*AP1 in the regulation of the *Tg*Rab5 membrane dynamics in *T. gondii*.

#### TgAP1 and the secretory pathway

The presence of an unusual TGN/ELC connected compartment could explain a role of *Tg*AP1 in constitutive secretion, such as described in plants. In plants, AP1 regulates the secretion of plasma membrane proteins from the hybrid TGN/EE directly or indirectly via the recycling pathway [27] [28]. Similarly, a major role of AP1 for membrane protein secretion has been also described in mammalian polarised cells. First, we found *Tg*AP1 present in numerous vesicles spread throughout the cytoplasm and close to the periphery. Furthermore, DDcMycµ1 parasites showed deformed more labile cortex correlated with a distorted morphology. Finally, daughter cell segregation seems to be impaired in *Tg*APµ1-KO parasites and DDcMycµ1. This might be due to a defect in membrane delivery at the interface of newly formed daughter cells. A similar role for AP1-dependent delivery of Golgi derived vesicles at the cleavage furrow of dividing cells, has been previously described in different organisms, such as *S. pombe* [41], *D. discoideum* [42], *C. elegans* embryo and plants [32], suggesting a conserved function for AP1 among various eukaryotic organisms.

Moreover, the fact that a unique ENTH-domain containing protein is expressed in *T. gondii* raised the question of the role of clathrin-mediated endocytosis at the plasma membrane, a question that is still a matter of debate. In particular, clathrin was found mainly localised at the TGN and in cytoplasmic vesicles and perturbing its function led to defects in Golgi duplication and ROP and MIC biogenesis [43]. However, it was recently shown that parasites depleted for the cathepsin CPL internalised GFP proteins by a still unknown mechanism [44]. The role of clathrin- or *Tg*AP2-mediated endocytosis in

this process was not investigated. Here we found that 1) the unique *T. gondii* epsin protein mostly co-localises with *Tg*AP1 at the TGN and in cytoplasmic vesicles and 2) by Co-IP, *Tg*EpsL interacts with the *Tg*AP1 complex but not the *Tg*AP2 complex, in spite of our ability to detect some of the *Tg*AP2 sub-units encoded in the parasite genome. Therefore, these findings argue against a clathrin- and epsin-mediated mechanism for protein internalisation at the parasite plasma membrane, but further experiments will be required to confirm this hypothesis. In particular, we are currently investigating further EpsL function by depleting the protein. Due to the unique organisation of the parasite cortex, that comprises 3 lipid bilayers (the plasma membrane and the inner membrane complex), it's likely that *T. gondii* uses alternative specific pathways for the internalisation of macromolecules compared to mammalian cells.

In conclusion, in plant cells, secretory and endocytic routes intersect at the hybrid trans-Golgi network (TGN)/early endosomes (EE), where cargos from both the anterograde and the retrograde pathways are further correctly sorted in a timely manner [34]. In particular, BFA treatment caused fragmentation of the Golgi apparatus and accumulation of so called "BFA bodies" positive for early endosomal markers and containing proteins endocytosed from the plasma membrane. In *T. gondii*, we also found a tight physical and functional association between the TGN and the ELC throughout the cell cycle. BFA treatment led to the dispersion of both, the TGN and the *Tg*Rab5A and *Tg*Rab7 positive compartment. Although tightly connected, these two compartments are distinct, as recently suggested by the study of the retromer function, where depletion of *Tg*Vps35 led to the retention of *Tg*SORTLR in the endosomal compartment by inhibition of its retrograde transport to the TGN [22]. Likewise, we found in *Tg*AP1-defective parasites that rhoptry proteins are retained in *Tg*Rab5A-positive vacuoles distinct from the TGN compartment. Because of the functional and structural similarities between the *T. gondii* and the plant trafficking system, one can envision that many vesicular trafficking pathways and the corresponding molecular regulatory mechanisms are conserved. Accordingly, we found that *Tg*AP1 might regulate secretion to the plasma membrane, an

activity that could regulate cell division, as well as Rab5-dependent trafficking pathways for ROP and MIC organelle biogenesis.

#### **Materials and Methods**

#### **Cloning strategies**

Genomic DNA was isolated from the Type I RH $\Delta$ Ku80 strain parasites using the Promega Wizard genomic DNA purification kit and used as template for PCR. The p5RT70 loxp-AP1 $\mu$ HA-loxp-YFP-HX plasmid was generated by a 3 step cloning. First, a 2kb fragment from the endogenous 3'UTR of *Tq*AP $\mu$ 1 (TGGT1 289770) was amplified using primers

GCGCGAGCTCACGGAGAAGGAACGAGGAGCAAAG and cloned into a unique Sacl site of the mother vector p5RT70loxPKillerRedloxPYFP-HX [30]. As a second step the coding sequence of the gene was amplified with a HA epitope tag added at the C terminus using primers GCGCCCTAGGATGGCGGGGGGGCGTCTGCGGTGT and

GCGCAGATCTCTAAGCGTAATCTGGAACATCGTATGGGTAGGAGAGTCTCAGTTGGTACTCTCCA and inserted into the plasmid using the restriction sites AvrII and BglII respectively. As a final step, a 2.5kb fragment from 5'UTR TgAPµ1 amplified the of was with the primers GCGCGGTACCCAAGTTCCCGTTTGTCCTGG and GCGCGGGCCCTCTTGGGACTGCAAGATCGACTG cloned using the sites KpnI and ApaI respectively. The DDcMycµ1 parasites were obtained using the ddFKBP over-expression system [31] as follows: The TgAPµ1 gene was amplified with the following primers: GCGCATGCATATGGCGGGGGGCGTCTGCG and

GCGCTTAATTAACTAGGAGAGTCTCAGTTGGTACTCTCCATTTTGAGTGATG and cloned into the pG12-Tub8-DD-mCherrycMyc-HXGPRT vector using the restriction sites Nsil and Pacl. The plasmid was then digested by AvrII and BgIII to remove the DD and mCherry fragments. The DD cassette was introduced resulting amplification PCR (F: again into the vector after by CTTTTAGATCTAAAATGGGAGTGCAGG, R: GCGCCCTAGGTTCCGGTTTTAGAAGCTCCAC) and ligation into the AvrII and BgIII sites.

Primers used to generate 3'-terminally tagged genes integrated at the endogenous locus (knock-in parasites) and produce recombinant proteins are indicated in the following table.

Plasmid	Primers (F: Forward; R: reverse)	Linearizatio
		n enzyme
pLic EPSL- cmyc (HXGPRT )	F: TACTTCCAATCCAATTTAATGCCCTCGTTCTCTCCTTCTCAGACGTT R: TCCTCCACTTCCAATTTTAGCGAACCCCGTCGTAGCAGGAGAT	Ncol
pLic µ1- HA (DHFR)	F: TACTTCCAATCCAATTTAATGCGGATCTTCCCTAGTTCGCGCCAGTCAC R:TCCTCCACTTCCAATTTTAGCGGAGAGTCTCAGTTGGTACTACTCTCCATTTTG AGT	SnaBl
pLic- Rab5- YFP (DHFR)	F: TACTTCCAATCCAATTTAATGCACTTTTGCCTCCACATGCACACC R: TCCTCCACTTCCAATTTTAGCGTGAGTGTCTCAGAAGGGAAGAACG	Eco47III
pLic σ1- HA (DHFR)	F: TACTTCCAATCCAATTTAATGCGTGATCCACCACTTTGTCGAGATCTTGG R: TCCTCCACTTCCAATTTTAGCGTCATGTAAGCTTGACTCCACCTTTAGTGTTGCTC	EcoRV
GST-βear	F: GGATCCGAGAACTCCTCTGCCGACAAGGACGTTTTCAGA R: GAATTCTCACGACCGTGGCGTCAGCC	
GST-γear	F: GGATCCTTTCCGCCGATGAATGTCTTGAACGAGGACG R: GAATTCTCACGCGAGGAGTCCCGCGG	

#### Transient transfection and Cas9-mediated gene disruption

The plasmid pTOXO\_Cas9-CRISPR corresponds to pUC57 carrying the C-terminally HA/GFP tagged *S. pyogenes Cas9* gene [47] fused to 2 nuclear localization sequences expressed under the control of the TUB8 promoter as well as the *Tg*U6 promoter driving the gRNA. Twenty mers-oligonucleotides (Forward: GCTGAGCAAAGACAGCCCTC) corresponding to the *Tg*EpsL gene (TGGT1\_214180) were cloned using Golden Gate strategy [48]. The ccdB positive-selection marker acts by killing the background of cells with no cloned DNA. The plasmid was synthesized and fully sequenced by GenScript (Singapour).

#### Parasite culture and transfection

Toxoplasma gondii Type I RHΔKu80ΔHXGPRT and DiCreΔKu80ΔHXGPRT parasites were grown on confluent Human Foreskin Fibroblast (HFF) cells (CCD-1112Sk (ATCC, CRL-2429<sup>™</sup>)) which were cultured in complete DMEM (gibcoLife Technologies) supplemented with 10% Fetal Bovine Serum (GibcoLife Technologies) and 1% Pen Strep (gibcoLife Technologies). To obtain the DDcMycµ1 parasites, 50µg of the pG12-Tub8-DD-cmyc-AP1µ1-HXGPRT plasmid was transfected in RHAKu80AHXGPRT parental strain by electroporation following standard procedures. To obtain the TgAPµ1-KO parasites, 50µg of loxP-AP1µHA-loxP-YFP-HXGPRT construct was transfected in the DiCreAKu80AHXGPRT strain parasites. Following transfection, in both cases the parasites were subjected to Mycophenolic acid/Xanthine drug selection and verified for the transfection efficiency by immunofluorescence analysis. Subsequently the non-clonal populations of parasites were subjected to cloning by serial dilution. For the TqAPµ1-KO clonal parasites, integration of the transgenic construct at the endogenous locus was verified by a genotyping PCR using a forward primer (GACGCGTTTCACTTCCTCGCTTCCTC) located upstream of the cloned 5'UTR, and a reverse primer (GTTTACGTCGCCGTCCAGCTCGAC) located on the YFP cassette. To obtain clonal knock-in parasites, 25 µg of plasmids were linearized over-night and transfected into the RH∆Ku80∆HXGPRT parental strain by electroporation followed by drug selection and cloning. Transient transfections were performed in  $10*10^6$  parasites with 50 µg of the following plasmids: HA–tagged *Tg*Rab5A (V. Carruthers) / cMyc-tagged TgRab7 (M. Meissner) / cMyc-tagged TgRab5 (M. Meissner); GalNac-YFP (D. Roos); GRASP-RFP (K. Hager) and parasites were allowed to invade HFF cells for 24 h prior analysis.

#### Western Blot:

Parasites were lysed in lysis buffer (NaCl 150mM, TrisHCl 20mM, EDTA 1mM, 1% TritonX100, protease inhibitors) and total proteins were subjected to electrophoresis in a 10% polyacrylamide gel. The proteins were transferred onto a nitrocellulose membrane (Amersham<sup>™</sup>Protran<sup>™</sup> 0.45µm

NC) by a standard western blot procedure. The membrane was blocked with 5% milk (non-fat milk powder dissolved in TNT buffer: 100mM Tris pH8.0, 150mM NaCl and 0.1% Tween20) and probed with primary antibodies diluted in the blocking buffer. The primary antibodies were followed by respective species specific secondary antibodies conjugated to HRP. The antibody incubations were followed by thorough washing using the TNT buffer. The membranes were visualized using ECL Western blotting substrate (Pierce).

#### Immunofluorescence assays (IFA)

When indicated, infected confluent HFF monolayers were incubated for 1 h with 5µM of Brefeldin A (Sigma-Aldrich) before fixation with 4 % paraformaldehyde (PFA) in phosphate buffered saline (PBS), for 20 minutes. After quenching with 50mM NH<sub>4</sub>Cl, the coverslips were permeabilized with 0.2% triton dissolved in 5% FBS-PBS for 30 minutes. The coverslips were then incubated with primary antibodies in 0.1%triton dissolved in 2%FBS-PBS or 0.05% Saponin for 1 h and then washed with PBS, followed by goat anti-rabbit or goat anti-mouse secondary antibodies conjugated to Alexa Fluor 488 or Alexa Fluor 594 (Molecular Probes, Invitrogen). Images were acquired using a Zeiss LSM880 confocal microscope. Antibodies used for IFA experiments are the following: rabbit anti-HA (Cell Signaling Technology), mouse anti-cMyc (Abcam), mouse anti-SAG1, rabbit anti-GAP45 (D. Soldati-Favre), mouse anti-MIC2 (V. Carruthers), rabbit anti-M2AP (V. Carruthers), mouse anti-MIC3 (J.F. Dubremetz), anti-MIC8 (D. Soldati-Favre), mouse anti-ROP 2-4, mouse anti-ROP5 and rabbit anti-ROP1 (J.F. Dubremetz), anti-VP1 (V. Carruthers), rat anti-TgSORTLR, Rabbit anti-tubulin (D. Roos); mouse anti-chromo1 (M. Gissot).

#### Structured Illumination Microscopy (SIM)

SIM was used to obtain high-resolution images using an ElyraPS1 microscope system (Zeiss) with a 100x oil-immersion lens (alpha Plan Apochromat 100x, NA 1.46, oil immersion) and a resolution of

120 nm along the x-y axis and 500 nm along the z-axis (PSF measured on 100 nm beads; Sampling voxel size:  $0,050\mu$ m\*0,050 $\mu$ m\*0,150  $\mu$ m). Three lasers (405, 488, and 561 nm) were used for excitation. SIM images were acquired with an EMCCD camera (Andor Technology Ltd, UK) and processed with ZEN software, where exposure times varied between 100 and 150 ms. Three-dimensional images were generated using a z-step of 150 nm (total thickness ~5  $\mu$ m). The acquisition was done sequentially using Zeiss Filter Sets 43HE, 38HE and BP 420-480. 15 frames were acquired to reconstruct one image (5 rotations x 3 phases, with a SIM Grating period of 51 $\mu$ m for the blue channel, 42  $\mu$ m for the green channel, 34 $\mu$ m for the red channel). 100 nm beads were imaged to measure the chromatic mis-alignment of our system (fit procedure by the Zen software); this parameter enabled correcting the alignment on each acquired multi-channel stack. Image reconstructions and co-localization quantification were determined with IMARIS software (Bitplane).

#### Intracellular growth assay

*Tg*APµ1-KO parasites were allowed to invade HFF monolayers for 3 h and treated with 50nM Rapamycin for 6 h. After 3 washes with warm medium, parasites were allowed to grow for additional 16 h before fixation with 4% PFA. For the DDcMycµ1 strain, parasites were inoculated onto HFF for 3h and treated with or without shield-1 (1µM) for 16 h, before fixation with 4% PFA. In both cases, intracellular parasites were counted after staining with anti-TgGAP45 antibodies. The numbers of parasites per vacuole were counted for more than 200 vacuoles for each condition performed in duplicate. Data are mean values  $\pm$  standard deviation (SD) from three independent biological experiments.

#### **Invasion assay**

Intracellular DDcmycµ1 transfected parasites induced with or without 1µM Shield for 16 h or intracellular  $TgAP\mu1$ -KO parasites induced with 50 nM rapamycin as described above were mechanically released from host HFF cells. Two million parasites were then allowed to adhere to host cell monolayers by incubation on ice for 30 min then shifted to 37°C for 45 min. Non adherent

parasites were washed with PBS followed by fixation with 4% PFA for 10min. The red-green invasion staining procedure was followed as described earlier [49]. Briefly, adherent external parasites were labeled with mouse anti-TgSAG1 antibodies, followed by secondary anti-mouse antibodies coupled to Alexa594. After cell permeabilisation with Triton 0.1% for 10 min, invaded intracellular parasites were detected using rabbit anti-TgGAP45 antibodies (D. Soldati-Favre) followed with secondary anti-rabbit antibodies coupled to Alexa488. At least, 300 parasites were counted for each condition performed in duplicate. Data are mean values ± standard deviation (SD) from three independent biological experiments.

#### Immunoprecipitation

For immunoprecipitation assays, a minimum of 0.6 billion parasites of pLIC-*Tg*APµ1-HA, pLIC-*Tg*EpsLcmyc / pLIC-*Tg*APµ1-HA, and DDcmycµ1 strains were lysed on ice for 30 min in modified RIPA buffer (50mM TrisHCl pH8.0, 2m MEDTA, 75m MNaCl, 0.65% NP40, 0.005%SDS, 0.5mM PMSF) and centrifuged at 14 000 rpm for 15 min to remove cell debris. Protein concentration was determined using the BCA protein assay kit (Pierce<sup>™</sup>). 500µg of total lysate were immunoprecipitated by binding to 50µl of anti-cmyc coated magnetic beads (Pierce<sup>™</sup>) or anti-HA agarose beads (Pierce<sup>™</sup>) overnight. After five washes of 10 mineach with modified RIPA buffer, bound proteins were eluted by boiling the samples in laemmeli buffer. Samples were then subjected to SDS PAGE and western blotting or gel-extracted for tryptic digestion and mass spectrometry analysis.

#### GST pull-down

The C-terminal ear appendage domain of the  $\beta$  (BAE) and  $\gamma$  (GAE) subunits were GST tagged by cloning into a pGEX6p3 vector (Pharmacia). Expression of GST-BAE and GST-GAE in BL21 competent cells was achieved by induction with 1mM IPTG at 37°C for 4 h. Bacteria lysates expressing GST-BAE , GST-GAE, and GST (control) were bound to 100ml of Protino Glutathione agarose 4B beads (Machery Nagel) in GST-lysis/binding buffer (Tris HCl (pH 7.6) 50mM, EDTA 1mM, EGTA1mM, 2-mercaptoethanol 10mM, NaCl 150mM, TritonX-100 0.5%, and 0.5mM PMSF) overnight at 4°C. The beads were washed 5 times with wash buffer A (Tris HCl (pH 7.6) 50mM, 2-mercaptoethanol 10mM,

NaCl 500mM, Triton 0.5% and 0.5mM PMSF) and 3 times with wash buffer B (Tris HCl (pH 7.6) 20mM, NaCl 150mM, NP40 0.65%, SDS 0.005%, 0.5mM PMSF) sequentially. Beads containing 150µg of the recombinant proteins and the control GST protein were incubated with a lysate from 0.4 billion pLIC-EPSL-cmyc / pLIC-m1-HA intracellular parasites overnight at 4°C. Parasites were lysed using modified RIPA (TrisHCl (pH8.0) 50mM, EDTA 2mM, NaCl 75mM, NP40 0.65%, SDS 0.005%, PMSF 0.5mM). After 3 washes with the lysis buffer, the proteins bound to the beads were eluted with 1x Laemelli blue buffer by boiling. The samples were subject to western blot and mass spectrometric analyses.

#### Mass spectrometry proteomic analysis

After denaturation at 100°C in 5% SDS, 5% βmercaptoethanol, 1 mM EDTA, 10% glycerol, 10 mM Tris buffer pH 8 for 3 min, protein samples were fractionated on a 10% acrylamide SDS-PAGE gel. The electrophoretic migration was stopped as soon as the protein sample entered 1 cm into the separating gel. The gel was briefly stained with Coomassie Blue, and five bands, containing the whole sample, was cut. In gel digestion of gel slices was performed as previously described [50]. An UltiMate 3000 RSLCnano System (Thermo Fisher Scientific) was used for separation of the protein digests. Peptides were automatically fractionated onto a commercial C18 reversed phase column (75 μm×150 mm, 2 μm particle, PepMap100 RSLC column, Thermo Fisher Scientific, temperature 35 °C). Trapping was performed during 4 min at 5µl/min, with solvent A (98 % H2O, 2% ACN and 0.1 % FA). Elution was performed using two solvents A (0,1 % FA in water) and B (0,1 % FA in ACN) at a flow rate of 300 nl/min. Gradient separation was 3 min at 5% B, 37 min from 5 % B to 30% B, 5 min to 80% B, and maintained for 5 min. The column was equilibrated for 10 min with 5% buffer B prior to the next sample analysis. The eluted peptides from the C18 column were analyzed by Q-Exactive instruments (Thermo Fisher Scientific). The electrospray voltage was 1.9 kV, and the capillary temperature was 275 °C. Full MS scans were acquired in the Orbitrap mass analyzer over m/z 300-1200 range with resolution 35,000 (m/z 200). The target value was 5.00E+05. Ten most intense peaks with charge state between 2 and 4 were fragmented in the HCD collision cell with normalized collision energy of 27%, and tandem mass spectrum was acquired in the Orbitrap mass analyzer with resolution 17,500 at m/z 200. The target value was 1.00E+05. The ion selection threshold was 5.0E+04 counts, and the maximum allowed ion accumulation times were 250 ms for full MS scans and 100 ms for tandem mass spectrum. Dynamic exclusion was set to 30 s.

#### Proteomic data analysis

Raw data collected during nanoLC-MS/MS analyses were processed and converted into \*.mgf peak list format with Proteome Discoverer 1.4 (Thermo Fisher Scientific). MS/MS data was interpreted using search engine Mascot (version 2.4.0, Matrix Science, London, UK) installed on a local server. Searches were performed with a tolerance on mass measurement of 0.2 Da for precursor and 0.2 Da for fragment ions, against a composite targetdecoy database (50620 total entries) built with 3 strains of *Toxoplasma gondii* ToxoDB.org database (strains ME49, GT1 and VEG, release 12.0, September 2014, 25264 entries) fused with the sequences of recombinant trypsin and a list of classical contaminants (46 entries). Cysteine carbamidomethylation, methionine oxidation, protein N-terminal acetylation and cysteine propionamidation were searched as variable modifications. Up to one trypsin missed cleavage was allowed.

#### Correlative light electron microscopy (CLEM)

Host cells were cultured on alphanumerical gridded-glass bottom dishes (**P35G-1.5-14-CGRD**, MatTek Corporation, Ashland, MA, USA) until 50% confluence was reached. Parasites were allowed to invade for 2 h, washed twice with warm medium, then induced for 6 h with Rapamycin 50n M, washed thrice with PBS and allowed to grow for additional 16hrs. Cells were then fixed with 4% PFA / 0.5% glutaraldehyde in PBS over-night. YFP-positive *Tg*APµ1-KO parasites were imaged using a Zeiss LSM880 confocal microscope and localized on the alphanumerical grid using transmitted light. After observation, cells were fixed with 2% glutaraldehyde in 0.1M sodium cacodylate buffer over-night. After washing with water, cells were sequentially stained with 1% osmium tetroxide reduced with 1.5% potassium hexacyanoferrate(III) for 1 hour, 1% thiocarbohydrazide for 30 minutes, 1% osmium

tetroxide, 1% uranyl acetate overnight at 4°C, and finally lead aspartate for 3 h. All stains were made in water, in the dark and at room temperature unless otherwise indicated. All stains were also washed with water. After staining, cells were dehydrated in graded ethanol solutions, infiltrated with epoxy resin and cured at 60°C for 48 h. After separation of the resin from the glass, cells of interest were relocated with the imprinted-alphanumerical grid at the surface of the resin. Small blocks of resin containing the cells of interest were prepared for sectioning parallel to the resin surface. Serial sections of 80 nm thickness were set down on carbon/formvar-coated slot grids. Sections were observed with a Hitachi H7500 TEM (Elexience, France), and images were acquired with a 1 Mpixel digital camera from AMT (Elexience, France).

#### Statistics

Means and SD were calculated in Excel. *P*-values were calculated in Excel using the Student's *t*-test assuming equal variance, unpaired samples and using two-tailed distribution.

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#### **Figure Legends**

#### Fig 1. TgAPµ1 localizes at the Trans-Golgi-Network and on secretory vesicles

**A**-Left: Western Blot (WB) showing the expression of the endogenous µ1 HA-tagged sub-unit of TgAP1 at the expected size of 49kDa in knock-in parasites (RH $\Delta$ kU80: parental strain). Actin was used as a loading control. B- Confocal microscopy images showing the localisation of µ1-HA (green) at the TGN together with TgSORTLR (red). Bar = 2µm. **C**- SIM image showing the partial co-localisation of  $\mathbb{P}SIM$  image showing TgSORTLR (red) in sub-regions of the Golgi apparatus. Bar: 1µm. **D**- SIM image showing the localisation of µ1-HA (green) in vesicles spread throughout the parasite cytoplasm and also present inproximity to the plasma membrane (arrows). Bar: 2µm. **E**- The co-localisation of µ1-HA with cis-Golgi (GRASP-RFP), trans-Golgi (GalNAc-GFP), and with endosomal compartments (TgRab5A-YFP) markers was examined by IFA and SIM microscopy. For each marker, a zoom on the Golgi region is shown (inserts). Bars: 2µm. F- Left: Cartoon showing the localisation of the different studied markers associated with the Golgi/ELC compartments. Graph depicting the percentage of co-localisation between µ1-HA and GalNAc-GFP, TgSORTLR and TgRab5A. µ1-HA displays the higher co-localisation with the TGN markers TgSORTLR and GalNAc-GFP, and is found slightly below the ELC compartment (TgRab5A).

#### Fig 2. The unique ENTH-domain containing protein of *T. gondii* is a key partner of *Tg*APµ1.

**B-** IFA showing the co-localisation of TgEpsL-cmyc (green) with the  $Tg\mu1$ -HA and TgSORTLR (red) at the TGN, analysed with confocal microscopy (upper panel) or SIM (lover panel). Bars: 2 $\mu$ m. **C**- Coimmunoprecipitation of  $Tg\mu1$ -HA with TgEpsL-cMyc in double KI parasites expressing both proteins at the endogenous level using an anti-cMyc antibody. No binding of  $\mu1$ -HA protein with the anticMyc antibody was detected in the control  $Tg\mu1$ -HA KI parasites. **C**- Reverse co-immunoprecipitation of  $Tg\mu1$ -HA with TgEpsL-cMyc in double KI parasites using an anti-HA antibody. **E**, **F**- A GST-pull down experiment with the GST-GAE and GST-BAE domains of TgAP1 was performed with a total lysate from  $TgEpsL-cMyc/Tg\mu1$ -HA double KI parasites. **E**: SDS-PAGE gel stained with coomassie blue showing the binding on glutathione beads of GST, GST-year and GST-βear at the expected size and in a similar quantity. **F**: WB analysis indicated the preferential binding of the TgEpsL-cMyc protein to the ear domain of the  $\gamma$ 1 sub-unit (GAE). A weak interaction of *Tg*SORTLR and the ear domain of the  $\beta$ sub-unit (BAE) was also detected. GST alone was used as a control. FT: Flow-Through.

#### Fig 3. CreLox-based strategy used to deplete TgAPµ1.

**A**- Scheme depicting the cloning strategy used to replace the endogenous μ1 locus by the LoxP-μ1-HA-LoxP insert. Upon rapamycin induction, the DiCre recombinase excised the LoxP flanked locus leading to YFP expression. Primers used to verify integration of the insert and its excision upon rapamycin incubation, are indicated. **B**- PCR confirming the integration of the LoxP-μ1-HA-LoxP insert at the endogenous *Tg*APμ1 locus resulting in the amplification of a band at 4,6Kb. Rapamycin induction resulted in the amplification of a supplementary band at 3.1 Kb corresponding to the 12% parasites with an excised locus. Primers used are depicted in A-. **C**- WB showing the expression of integrated LoxP-μ1-HA protein at the expected size in a clonal population (RHΔKu80Dicre: parental strain). ROP 2-4 was used as a loading control. Of note, expression of rhoptry proteins was not affected after integration of *Tg*μ1-HA (green) at the TGN, after integration of the sequence flanked by the LoxP sites at the endogenous locus, together with *Tg*SORTLR (red) in RHΔKu80DiCre parasites. Bar: 2μm. **Lower panel:** IFA showing the absence of μ1-HA signal in YFP positive parasites upon rapamycin treatment. Bars: 5μm.

#### Fig 4. *Tg*APµ1 knock-out impairs microneme formation.

**A-** IFA showing the localisation of MIC8, MIC3, MIC2 and M2AP proteins (red) in control and  $TgAP\mu1$ -KO parasites. MIC8 accumulates in the parasite Golgi, confirmed by its co-localisation with TgSORTLR while MIC3 is secreted into the parasitophorous vacuolar space (arrow; parasite contours are marked with IMC1). MIC2 shows a preferential apical localisation, while lateral micronemes are weakly detected. M2AP localisation was not found affected in  $TgAP\mu1$ -KO parasites. Bar:  $2\mu m$ .

**Fig 5. A**- Confocal images showing the localisation of rhoptry (ROP2-4) and pro-ROP4 proteins (red) in control (YFP-negative vacuoles) and *Tg*APµ1-KO parasites (YFP-positive vacuoles) in relation with the TGN marker *Tg*SORTLR and the IMC marker IMC1 (white). In *Tg*APµ1-KO parasites, mature rhoptries were found fragmented within the cytosol (upper panel) or in the vacuolar space (middle panel), while immature pro-ROP4 proteins were found re-routed towards the vacuolar space and residual body (lower panel). Bar: 2µm. **B**- Graph indicating the percentage of vacuoles displaying a normal apical rhoptries localization in control and *Tg*APµ1-KO parasites. Data are presented as mean ± SEM, p<0.001 (student t-test). **C**- Graph indicating the percentage of vacuoles positive for the immature protein Pro-ROP4 staining in control and *Tg*APµ1-KO parasites. Data are presented as mean ± SEM, p<0.01 (student t-test).**D**- Graph depicting the percentage of invaded parasites after 45 min

incubation with host cells of mechanically egressed parasites for control (YFP-neg) and  $TgAP\mu1$ -KO (YFP-pos) parasites. Data are presented as mean ± SEM, p<0.001 (student t-test).

#### Fig 6. The inducible over-expression of $TgAP\mu1$ only perturbed rhoptry formation.

**A-** WB showing the accumulation of DDcMycµ1 protein upon Shield-1 treatment for the indicated time periods. The protein eno2 was used as loading control. **B-** IFA analysis of the localisation of DDcMycµ1 protein (red) in transfected RH parasites. Bar: 2µm. DDcMycµ1 was detected at the Golgi apparatus (co-localisation with *Tg*SORTLR (green) and in vesicles spread out through the cytoplasm and accumulating at the basal pole of the parasites. **C-** IFA showing the localisation of ROP1, ROP5, *Tg*SORTLR (upper panels) and MIC2, MIC3 and MIC8 (lower panels) in control (parental strain RH incubated with Shield-1) and shield-1 induced DDcMycµ1 parasites. Bars: 1µm. **D-** IFA showing the co-localisation of ROP2-4 and proROP4 proteins (upper panel) and proROP4 with *Tg*Rab5A-HA (lower panels) in control (parental strain RH incubated with Shield-1) and shield strain RH incubated with Shield-1 induced DDcMycµ1 parasites. Bars: 2µm. **E-** Invasion assay. Graph depicting the percentage of invaded parasites after 45 min incubation of mechanically egressed parasites with host cells for the parental strain and DDcMycµ1 over-expressing parasites. Data are presented as mean ± SD, p<0.01, (student t-test).

## Fig 7. TgAP1 regulates $T_g$ Rab5A compartment dynamics and rhoptry maturation at a post-Golgi level

**A-** IFA followed by SIM data acquisition showing the localisation of pro-ROP4 (red) and *Tg*Rab5-HA proteins (green) in control RH parental strain (upper panel) and DDcMycµ1 over-expressing parasites (lower panel) treated with Shield-1. Bars: 2µm. **B-** Upper panel: SIM microscopy images showing the co-distribution of Rab5-positive vesicles surrounding pro-ROP4 vacuolar compartments in RH parental strain, while TGN-distant pre-ROP compartments were negative for *Tg*Rab5A staining (arrow). Lower panel: SIM images showing the presence of *Tg*Rab5A-positive enlarged vacuolar compartments containing or not pro-ROP proteins in DDcMycµ1 parasites. Bars: 500 nm. **C**-Quantification of the percentage of pro-ROP4 positive vacuoles displaying also a *Tg*Rab5A signal at their limiting membrane in RH and DDcMycµ1 parasites. Data are presented as mean ± SD (n= 50 parasites), p<0,001 (student t-test).

#### Fig 8. TgAP1 regulates the parasite division process at a late stage of cytokinesis.

**A**, **B**- Intracellular growth assay performed on *DDcMycmu1* parasites after  $\pm$  Shield-1 induction for 16 h (A) and *TgAPµ1-KO* parasites after rapamycin treatment (B), revealed defects in cell replication. The graphs depict the percentage of vacuoles containing 2, 4, 8, or 16 parasites. Data are presented as mean  $\pm$  SD. The confocal image on the right shows a disorganized *TgAPµ1-KO* vacuole. **C- upper panel:** Confocal microscopy images showing the localisation of tubulin and chromo1 in control

parasites at the different stages of the cell cycle: G1 phase: 1nucleus/1centromere positive for Chromo1 (1N/1CC); M phase: Centromer have duplicated (1N/2CC); Late M phase: Daughter nuclei have been separated by the daughter scaffolds (2N/2CC); late cytokinesis: At the end of cell segregation, chromo1 is no longer detected at the centromeres but localised in some vacuoles at the residual body: 2N/OCC. **Lower panel**: Confocal images showing the duplication of the centromeres (Chromo1) and the centrosomes (centrin 1) in *Tg*APµ1-KO parasites (YFP positive). Bars: 1µm. **D**-Graph depicting the percentage of vacuoles observed in each of the different stages of the cell cycle described above in control and *Tg*APµ1-KO parasites. Data are presented as mean  $\pm$  SEM p<0,01 (student t-test). **E**- Confocal microscopy images showing the formation of daughter buds (IMC3 and IMC1) in control parasites and *Tg*APµ1-KO parasites. Of note, parasite polarity is preserved as seen by the localization of MIC2 (red) at the apex of the budding daughter cells. Bars: 2µm. **G**- Confocal microscopy images showing the formation (red) in control parasites and *Tg*APµ1-KO parasites, MORN1 is normally observed at the basal pole of parasites, however often parasites appear to stay bound by their basal pole (arrow). Bars: 2µm.

**Fig 9. CLEM microscopy confirmed the** *Tg***APμ1-KO parasite defects in rhoptry and microneme formation and in cell division**. *Tg*APμ1-KO parasites were analysed by Correlative Light Electron Microscopy (CLEM) as described in Methods. Electron microscopy images obtained after serial sectioning showed correct organisation of replicating parasites in rosette-like structure in control YFP-neg parasites (A). The parasites display typical rhoptry and microneme organelles at the apical pole (B). *Tg*APμ1-KO parasites exhibit a strong defect in gross and cortex integrity (C, D), an accumulation of intracellular big–sized vacuoles at the apical pole (E) while apical micronemes were still detected (F); and a swollen vesiculated Golgi (G). In less affected vacuoles, parasites displayed membranous extensions at their basal pole linking distant parasites (H). Bars: A, C: 2μm D, H: 1μm and B, E, F, G: 500nm.

#### <u>**Table I**</u>: IP *Тд*µ1-НА

			Total nu unique pept	umber of ides / spectra
Protein name	Accession numbers	Molecular weight (Da)	ΙΡ-1 μ1-ΗΑ	ΙΡ-2 μ1-ΗΑ
mu1 adaptin	TGGT1_289770	48 918	11/55	8/16
gamma1 adaptin	TGGT1_313670	107 036	20/53	24/77
beta adaptin	TGGT1_240870	101 920	18/47	19/76
sigma1 adaptin	TGGT1_270370	19 679	2/4	1/2

clathrin heavy chain	TGGT1_290950	194 480	4/6	1/1
ENTH-domain containing protein	TGGT1_214180	65 903	1/1	1/1

#### Table II: IP TgEpsL-cmyc

	Accession	Molecular	Total number of unique peptides / spectra	
Protein name	numbers	weight(Da)	Control:	EpsL-cmyc/
			μιπτ	μ1 Π/
ENTH domain-containing protein	TGGT1_214180	65 903	3⁄4	15/76
beta adaptin	TGGT1_240870	101 920	/	19/28
gamma1 adaptin	TGGT1_313670	107 036	2/3	17/33
mu1 adaptin	TGGT1_289770	48 918	/	9/11
ADP ribosylation factor ARF1	TGGT1_276140	61 745	2/2	6/12

#### Table III: IP DDcMycµ1

Protein name	accession numbers	molecular weight (Da)	Number of peptides	Number of spectra
gamma 1 adaptin	TGGT1_313670	107 036	49	189
beta adaptin	TGGT1_240870	101 920	51	160
Sigma1 adaptin	TGGT1_270370	19 679	9	63
ENTH domain-containing protein	TGGT1_214180	65 903	4	4

#### **Supplementary Figures**

**Fig S1. A-** IFA showing the co-localisation of  $Tg\mu$ 1-HA (red) and cMyc-TgRab5 (green) at the duplicated Golgi during the G1/S phase of the cell cycle. Bar: 2 $\mu$ m. **B-**  $\mu$ 1-HA KI parasites or RH parasites transiently transfected with TgRab5-HA and TgRab7-HA were treated (or not) with Brefeldin A for 1 h before fixation and processing for IFA. Confocal images showing the dispersion of  $\mu$ 1-HA, TgRab5 and TgRab7 positive endosomal compartments (green) in vesicles and aggregates. Bar: 2 $\mu$ m.

**Fig S2. A**- Left: WB showing the expression of the  $\mu$ 1-*Tg*HA sub-unit of *Tg*AP1 at the expected size (20 kDa) in KI parasites (using anti-HA antibodies) and not in the parental RH $\Delta$ Ku80 strain. **B**: IFA showing

the localisation of the  $Tg\mu$ 1-HA sub-unit at the TGN, together with TgSORTLR. Bars: 1µm. **C**-Sequence alignment of the unique *T. gondii* ENTH-domain containing protein (TGGT1\_214180) with *Human* epsin1 (*Hs*, UniProtKB-Q9Y6I3) and epsinR (CLINT1/Epsin4, UniProtKB- Q14677), with *Arabidopsis Thaliana* epsinR2 (*At*, UniProtKB - Q67YI9) and *Plasmodium falciparum* (*Pf*) unique ENTH-domain containing protein (PF3D7\_1245800). A scheme illustrating the positions of the identified conserved domains in *Tg*EpsL, such as the ENTH domain, the clathrin binding site and the NPL motif (the latter being predicted to mediate the association of epsin proteins with clathrin adaptor complexes), is also shown.

**Fig S3.** Schemes illustrating the conserved identified domains of the AP1 $\mu$  (TGGT1\_240870) and AP1 $\mu$  (TGGT1\_313670) sub-units of the *Tg*AP1 complex, including the N-terminal Adaptin domain, the clathrin binding sites present in the hinge domain and the C-terminal appendage ear (AE) domains. Sequence alignments of the GAE and BAE domains of *T. gondii* AP1 (*Tg*) with the corresponding sequences found in the AP1 complex of *Plasmodium falciparum* (*Pf*), *P. berghei* (*Pb*), *Arabidopsis Thaliana* (*At*) and Human (Hs) are also shown. The accession numbers of analysed proteins are indicated in supplementary Tables I and II.

**Fig S4**. **A**- SIM microscopy images showing the localisation of  $Tg\mu$ 1-HA (green) and Pro-M2AP (left) or Pro-MIC3 (right) in  $Tg\mu$ 1-HA KI parasites. No co-localisation between  $Tg\mu$ 1-HA and Pro-M2AP was observed as opposed to MIC3, which shows a strong co-localisation with Tg2gHA and Pro-M2AP was observe**B**- IFA showing the localisation of TgSORTLR in YFP positive  $TgAP\mu$ 1-KO parasites and YFP negative control parasites. TgSORTLR is not mis-localised in  $TgAP\mu$ 1-KO, but the Golgi appears swollen compared to control YFP-negative parasites (see also Figure 8). Bar:  $2\mu$ m. **C**- IFA showing the localisation of GRA3 and GRA6 proteins in  $TgAP\mu$ 1-KO YFP positive parasites. No defect in dense granule biogenesis was observed upon  $TgAP\mu$ 1 depletion. Bar:  $1\mu$ m.

**Fig S5. A-** IFA was performed to study the localisation of  $\mu$ 1-HA protein in RH $\Delta$ Ku80 parasites transiently transfected with the DDcMyc $\mu$ 1 protein and induced with shield-1 for 16 h. The endogenous  $\mu$ 1-HA sub-unit was found correctly localised at the Golgi. Bar: 2 $\mu$ m. **B-** Transmission Electron Microscopy images showing the normal distribution of parasites in rosette-like structures, as well as the formation of mature rhoptries (Rh) and micronemes (Mi) anchored at the apical pole in control parasites (A-B). In DDcMyc $\mu$ 1 (C-F), rhoptries could not be detected in opposite to micronemes. Numerous giant lucent vacuoles (V) were also observed. In addition, the parasites were found disorganised within the vacuole with a distorded morphology and labile cortex (arrows). Bars: 500nm.

**Fig S6. A, B-** Strategy used for CLEM microscopy. HFF cells were allowed to grow (50% confluent) on alphanumerical gridded coverslips. Confocal microscopy images were taken (40X objective) to spot the YFP positive parasites corresponding to  $TgAP\mu1$ -KO parasites. A mosaic of 8\*8 microscopy fields centred on the parasites of interest was then acquired to determine the vacuole position on the grid as illustrated in A and B. The  $TgAP\mu1$ -KO vacuole indicated in A (position BR) corresponds to EM images shown in Figure 9 (D, E, F), while the  $TgAP\mu1$ -KO vacuole indicated in B (position 3T) corresponds to the EM images shown in Fig. 9C, G, H. The non-YFP vacuole detected in the 3T zone was used as the negative control shown in Fig. 9A and B.

Name	Accession number
TgAP1Gamma (T. gondii)	TGGT1_313670
PfAP1Gamma (P. falciparum)	PF3D7_1455500
PbAP1Gamma (P. berghei Anka)	PBANKA_1319200
HsAP1Gamma (Homo sapiens)	UniProtKB - O43747
AtAP1Gamma (Arabidopsis thaliana)	UniProtKB - Q84K16

Supplementary	table I: accession	numbers of the genes	used for the seau	Jence alignment sho	wed in Figure 2
		mannoers of the genes			

Supplementary table II: accession numbers of	f the genes used for the seq	juence alignment showed in Figure 2
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Name	Accession number
TgAP1β (T. gondii)	TGGT1_240870
PfAP1β (P. falciparum)	PF3D7_0528100
PbAP1β (P. berghei Anka)	PBANKA_1242700
HsAP1β (Homo sapiens)	UniProtKB - Q10567
AtAP1β (Arabidopsis thaliana)	UniProtKB - O81742

Supplementary movie: SM1

3D-SIM image reconstruction showing the localisation of the TgRab5A compartment (green) compared to the TGN localised  $TgAP\mu1$  (red). A KI parasite line expressing TgRab5-YFP under the native promoter was used for the study.

#### Supplementary movie: SM2

3D-SIM image reconstruction showing the localisation of the *Tg*Rab5A compartment (green) compared to the TGN marker *Tg*SORTLR (red). A KI parasite line expressing *Tg*Rab5-YFP under the native promoter was used for the study.



Figure 2









В

## Figure 4



### Figure 5












RH

DDcMycµ1





% of ProROP4/Rab5A vesicles 0 07 09 08 00

в

\_\_\_\_\_\_ ≡ RH +S \_\_\_\_\_ ■ DDcMycµ1 +S

146









## Figure S3





















# **III-2.** *Toxoplasma gondii* **Rab11A** regulates dense granule secretion during host invasion and parasite replication.

### III-2.1. TgRab11A localization during T. gondi cell cycle

In this study we have made use of the stabilizable ectopic over-expression of  $T_g$ Rab11A (ToxoDB Gene ID: TGGT1\_289680) in its wild type form to examine the localization of the protein. Briefly, the construct has Rab11A N-terminally tagged with ddFKBP, mCherry and cmyc, all placed under an  $\alpha$ -tubulin based p5RT70 constitutive promoter (the plasmid is a kind gift from Dr Markus Meissner) (Herm-Gotz et al., 2007; Agop-Nersesian et al., 2009). Upon incubation with the synthetic ligand Shield-1, the protein under the influence of the destabilization domain ddFKBP is no longer targeted to the proteasome for degradation and therefore accumulates rapidly (2 hours) in the parasite (Figure 1A). Moderate stabilized overexpression of the protein was achieved by treating the parasites with addition of 0.6µM Shield-1 to the growth medium. This working concentration of Shield-1 was defined according to the absence of a defect in cell division that we observed with a more intense over-expression of the WT protein. By immunofluorescence assay (IFA) in fixed parasites, we found that Rab11A localizes at the TGN, demonstrated by its co-localization with the clathrin adaptor complex AP1 subunit  $\mu$  (Figure 1B). To achieve this, we transfected the DDmCherrycMycRab11A construct in a stable knock-in parasite line expressing APµ1 tagged with a HA epitope (Chapter II-1), and established a clonal population of parasites. Apart from the TGN, Rab11A was also seen in large vesicles across the cytoplasm, particularly concentrated at the apical and basal poles of the parasite (Figure 1B). These regions were previously suggested to be sites that favor endo-exocytic events, as the Inner Membrane Complex (IMC), which possibly restrains the exchanges with the external environment, is interrupted at those sites. Co-localization experiments with the endosomal-like compartment (ELC) marker Rab5 showed that Rab11A localized just beneath the Rab5A-positive compartment, in agreement with the similar localization of AP1 previously observed (chapter II-1). Of note, a detailed characterization of Rab11A localization in the parasite is currently performed using super-resolution microscopy SIM. In particular, we are exploring Rab11A co-localisation with Cis-Golgi (GRASP, ERD), Trans-Golgi (GalNac, SORTLR), endosomal (Rab7 and Rab5), rhoptry (RON4, ROP2-4), micronemes (MIC2, MIC3), conoid (CAM1), and basal complex (MORN1) markers.

Next, in order to confirm that the localization of the endogenous protein is similar to the one observed for the overexpressed Rab11A, we raised a specific polyclonal mouse antibody against Rab11A. We first confirmed the reactivity and specificity of the antibody on western blots. While against the wild type RHAKu80 parasite lysate a single band corresponding to 25kDa was detected, in the lysate of parasites expressing DDmCherrycmycRab11A, a band at 67kDa representing the extra copy of the recombinant protein, was additionally seen when induced with Shield-1 (Figure 1C). A faint expression was also detected in uninduced parasites reflecting the inherent leakiness of this system. Using this antibody, we observed by IFA a similar localization of endogenous Rab11A compared to over-expressed DDmCherrycmyRab11A in replicating parasites. In addition, we found that the endogenous protein displays a dynamic distribution as its localization pattern changed between the different parasite division stages, suggesting pleiotropic roles for Rab11A in T. gondii. In G1 phase, Rab11A is present at the TGN and cytoplasmic vesicles, particularly enriched at the apex and basal pole of the parasite. During mitosis and beginning of cytokinesis, Rab11A was observed at the tips of budding daughter parasites (co-localization with the IMC marker, IMC3, will be performed). At the end of the cytokinesis process, Rab11A was found concentrated at the basal pole of the segregating daughter parasites (Fig 1B). Of note, this localization is reminiscent of the known role of Rab11 in cytokinesis in other Eukaryotes, in particular for the final step of furrow cleavage ingression (Horgan, C.P. et al., 2003). Furthermore, we did not find a clear co-localization of Rab11A with rhoptries as previously suggested (Bradley, P.J. et al., 2005; Agop-Nersesian, C. et al., 2009), though we cannot exclude that this co-localization is transient or discrete, in particular at the rhoptry neck, which also localized at the apex of the parasite (co-localisation with the rhoptry neck protein RON4 will be performed).



Figure 1. TgRab11A localization and protein expression

(A) Schematic representation of the DD stabilizable over-expression construct containing the Rab11A protein and western blot showing that the recombinant protein expression is stabilized by Shield-1 treatment  $(0,6\mu M)$  as early as two hours. (B). Upper panel: IFA experiment showing the colocalization of DDmCherrycmycRab11A (following induction with 0.6µM Shield-1) with the Clathrin Adaptor complex AP1 subunit u at the TGN (arrow). One can also notice the presence of Rab11A in cytoplasmic vesicles distributed predominantly at the poles (arrow heads). Lower panel: The partial co-localization of DDmCherrycmycRab11A with the endosome like compartment (ELC) marker Rab5A is presented. The magnified regions indicated by a white insert show that Rab11A localises at the TGN and is closely juxtaposed to the Rab5A positive ELC. Scale bars: 5um(C) Western blot showing that the polyclonal Rab11A antibody recognized the endogenously expressed Rab11A in wildtype RHAKu80 (band at the expected size of 25kDa) and both uninduced (-S) and Shield-1 induced (+S) transgenic parasites. In the lysates of parasites expressing the stabilized ectopic copy of Rab11A, a band at 67kDa is additionally detected in presence of Shield-1 (+S). The lower weak bands are result of degradation. (D) TgRab11A specific polyclonal antibody recognizes similar localization pattern as the stabilized over-expression during G1 phase of cell cycle (arrow head for vesicle and arrow for TGN). During mitosis and end stage cytokinesis phases, Rab11A localizes at the tips of budding daughter parasites (arrow) and at the basal pole residual body (arrow), respectively. Scale bar: 5µm.

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### III-2.2. Dynamics of Rab11A positive vesicles by live imaging in replicating parasites

To study in detail the dynamic localisation of Rab11A, we adopted live imaging. Using spinning disk confocal microscopy, we were able to capture the dynamic motion of Rab11A positive vesicles across the parasite cytoplasm in intracellular replicating parasites. Similar to the staining pattern obtained in fixed parasites, we observed that Rab11A partially localised at the Golgi region. In addition, a rapid bi-directional movement of Rab11A-positive vesicles was also seen between the apical and basal poles. These vesicles are frequently detected as following the lateral sides of the parasite, suggesting an actin-based movement related to the glideosome. In addition, dynamic movements of vesicles could be detected between the Golgi area and the apical tip of the parasite. Such a vesicular motion pattern, in particular between the TGN and the apical parasite surface, suggested that the protein is likely involved in the trafficking of secretory cargo within the parasite cytoplasm and to the cell surface, similar to what is widely known on Rab11 from studies on mammalian cells and other lower eukaryotes.



#### Figure 2. Live imaging of Rab11A positive vesicles in intracellular replicating parasites

(A) Two sets of time frames chosen from a movie captured using spinning disk microscopy show the movements of TgRab11A positive vesicles across the cytoplasm of intracellular parasites. The two panels show two different parasitophorous vacuoles (see insert boxes indicated on the whole field view). In the upper panel, one vesicle is tracked moving from the apex to the base of one of the parasites, and vice versa, in the lower panel (from base to apex) (arrows). Scale bar:  $2\mu m$  and  $5\mu m$  (whole field view).

# III-2.3. GDP locked dominant negative mutant of Rab11A (Rab11 $A_{DN}$ ) inhibits Dense Granule secretion and GT1 delivery to the plasma membrane

In parallel, we performed experiments to study in detail the functions of Rab11A. A dominant negative mutant of the protein (Rab11A<sub>DN</sub>), harboring a point mutation N126I in the functional GTPase domain, used previously by Dr Meissner's lab (Herm-Gotz, A., et al., 2007), was over-expressed in order to affect Rab11A activity. As previously observed (Agop-Nersesian, C. et al., 2009), we also confirmed that parasites over-expressing Rab11A<sub>DN</sub> displayed a striking defect in replication in comparison to Rab11A<sub>WT</sub> (not shown). Based on our hypothesis that Rab11A could contribute to secretory events, we investigated different trafficking pathways in parasites expressing Rab11A<sub>DN</sub>. It has been previously shown that Rab11A is not involved in rhoptry and microneme biogenesis (Agop-Nersesian, C. et al., 2009). At 4 hours post-invasion, we observed by IFA that Rab11A<sub>DN</sub> parasites showed normal rhoptries and micronemes organelles, correctly positioned at the apex and periphery of the parasites, respectively (Figure 3A). Western blot analysis also confirmed that there was no obvious defect in ROP and MIC protein level at this early time point post-invasion (Figure 3B). However, at later time points when the division process was drastically affected (24 hours post-invasion), IFA showed that the micronemes are partially mis-localized in the cytoplasm and the rhoptries appeared as fragmented compartments spread in the parasite cytoplasm (Figure 3C). Western blot analysis also indicated a defect in ROP and MIC protein synthesis and proteolytic processing (Figure 3D). The expression level of MIC5 clearly dropped in parasites expressing Rab11A<sub>DN</sub> parasites. ROP4 maturation appeared to be impaired as we observed an accumulation of the immature pro-ROP4 protein and a decrease in the level of mature ROP4 proteins in Rab11A<sub>DN</sub> compared to uninduced parasites.

We believe that this observed defect in ROP and MIC biogenesis at later time of replication is an indirect consequence of the cell division problem. In particular this defect is probably caused by the loss of cell polarity that might impair ROP and MIC organelle anchoring at the apex of the parasite and thereby organelle maturation. However, this point should be clearly demonstrated by additional experiments. In particular, we will carefully monitor ROP and MIC organelle formation during the first cell cycle division (8 to 10 hours post-invasion). At this stage, formation of the two first daughters IMC is not perturbed (only the recruitment of late glideosome components GAP45) and therefore cell polarity is preserved.



**Figure 3.** Micronemes and Rhoptry defects in Rab11A<sub>DN</sub> parasites. (A) Rab11A<sub>DN</sub> parasites induced with 1µM Shield-1 for 4 hours following invasion are compared with uninduced parasites. The formation of micronemes, labelled with anti-MIC5 antibodies and rhoptries labeled with anti-ROP4 antibodies, seemed unaffected at this early time post-invasion (see arrows). Scale bars = 2µm. (B) Western blot showing no differences in the level of expression of MIC5 and ROP4 proteins between uninduced and induced Rab11ADN parasites at 4 hours. Eno2 was used as a loading control. (C) IFA experiments were performed in Rab11A<sub>DN</sub> parasites that were allowed to grow for 24 hours without (-S) and with Shield-1 (+S) in host HFF cells. MIC5 reaches its correct apical localization (see arrow). ROP4 protein labeling indicates the presence of atrophied or fragmented rhoptries (see arrow) in Rab11ADN parasites induced with Shield-1. Scale bars= 2µm. (D) Western blots performed using lysates of Rab11A<sub>DN</sub> parasites grown for 24 hours with and without Shield-1 induction showing a drop in the level expression of MIC5 and ROP4 proteins. In the case of ROP4, the unprocessed pro-form accumulated in parasites induced with Shield-1. Eno2 was used as a loading control.

After examining ROP and MIC formation, we investigated the role of Rab11A in the constitutive secretory pathway of *T. gondii*. The so called "constitutive" secretory pathway is defined as the pathway regulating secretion of proteins to the parasite plasma membrane or export in the vacuolar space during intracellular parasite replication. A study on the GPI-anchored surface antigen SAG1, showed that depletion of its GPI anchor, rerouted the protein into the dense granule secretory pathway (Striepen, B. et al., 1998). From this evidence, the dense granules were proposed to represent the default pathway for protein exit from the

parasite in a non-regulated manner, by opposition to ROP and MIC protein release triggered during parasite entry. Of note, a recent study employed the fluorescently labeled SAG1 devoid of its GPI anchor to track the movement of dense granule vesicles during intravacuolar replication. This study indicated that DG move in acto-myosin dependent (microtubule independent), diffusive-like (in the cytoplasm) as well as directed (at the cell cortex) movements (Heaslip, A. et al., 2016). The DG motion described in this study was very similar to what we observed for Rab11A-positive vesicles. Therefore, together with the role of Rab11A in SAG1 delivery to the parasite surface (Agop-Nersesian et al., 2009), we hypothesized that Rab11A might possibly play a role in dense granule trafficking and secretion.

To address this question, we made used of our parasites defective for Rab11A activity. Following treatment with 1µM Shield-1 for 16 hours on intracellular replicating Rab11A<sub>DN</sub> parasites, IFA was performed on fixed coverslips. First, we monitored DG secretion in the vacuolar space following a selective permeabilization protocol. We selectively permeabilized the parasitophorous vacuolar membrane (PVM) using a low dose of saponin, which does not permeabilize the plasma membrane (Figure 4A and 4B). Using this method, we quantified the percentage of GRA-positive vacuoles and confirmed the drastic decrease of GRA1 and GRA3 protein secretion into the parasitophorous vacuolar space (Figure 4C). Following triton permeabilization of both the PVM and the parasite plasma membrane (PM), we conclusively observed the arrest of GRA proteins in the parasite cytoplasm (Figure 4A). Similar results were obtained for GRA5, GRA2 and GRA6 (not shown). We also examined GRA16, a protein shown to be secreted into the vacuolar space before reaching the host cell nucleus. GRA16 secretion was monitored by generating Knock-In parasites expressing a HA tagged GRA16 under the natural promotor (a gift from Dr A. Hakimi, Grenoble) in the established clonal populations of Rab11A<sub>WT</sub> and Rab11A<sub>DN</sub>. We found that GRA16 secretion was inhibited upon the over-expression of Rab11A<sub>DN</sub>, never reaching the host cell nuclei (Figure 4D). Of note, this experiment is under quantification for the number of GRA16-positive nuclei after Rab11AWT or Rab11ADN overexpressing parasite infection.

Together, these observations demonstrated a novel role of Rab11A, not only in SAG1 delivery to the surface (Agop-Nersesian, C. et al., 2009), but also in DG protein secretion in the vacuolar space. Importantly, we did not observe an accumulation of GRA proteins at the Golgi or the endosome-like compartments in Rab11A<sub>DN</sub> parasites, suggesting that Rab11A is not involved in protein exit from the Rab11A-associated TGN compartment.



#### Figure 4.TgRab11A influences secretion of Dense granule proteins

(A) Selective permeabilization (0.03% of saponin) of the parasitophorous vacuolar membrane (PVM) and not the parasite plasma membrane revealed the DG proteins secreted into the vacuolar space and at the PVM in Rab11A<sub>WT</sub> but not in Rab11A<sub>DN</sub> parasites. Upon cell permeabilization with 0.2% triton, GRA1 and GRA3 proteins were detected as retained within the cytoplasm of Rab11A<sub>DN</sub> expressing parasites. Scale bars:  $5\mu$ m. (B) The graph indicates the percentage of parasitophorous vacuoles positive for GRA1 and GRA3 labeling in the vacuolar space following saponin permeabilization. 100 vacuoles for each strain were counted in each of the two experimental duplicates. 3 independent experiments were performed. Error bars represent SEM (Standart Error Mean). (D) IFA showing the localization of GRA16 protein in intracellular parasites 16 hours post-invasion. The GRA16 protein, which normally reaches the host cell nucleus and vacuolar space (right insert, arrow) in Rab11AWT expressing parasites, remains dispersed within the cytoplasm in Rab11ADN parasites (right insert, arrow) and is never detected in host cell nuclei. Scale bars:  $5\mu$ m.

However, all the above observations were made in parasites that had been allowed to grow for 16 hours. One could argue that the GRA secretion inhibition is due to an indirect defect in the parasite cortical integrity caused by the IMC formation blockage. Similarly, it has been shown that GRA proteins accumulate in the PVM or vacuolar space in a dosedependent manner while parasites replicate and increase in number within the vacuole. Therefore, in order to rule out these possibilities, we compared secretion of dense granules at the end of the first cycle of division in Rab11A<sub>WT</sub> versus Rab11A<sub>DN</sub> parasites, before drastic differences in replication or parasite number is observed between these two lines. We confirmed that contrary to ROP and MIC formation, GRA secretion was completely blocked early after invasion in Rab11A<sub>DN</sub> parasites, compared to Rab11A<sub>WT</sub> parasites where GRA proteins were clearly detected in the PV space or at the PVM (Figure 5A). We also observed a secretion defect by western blot (Figure 5A and 5B). The western blots revealed an increase in the protein levels for GRA1 and GRA3 contained in intracellular parasites expressing Rab11A<sub>DN</sub> and induced for 8 hours, likely reflecting the inhibition of secretion of dense granules in these parasites (this experiment has to be repeated).

Further, the previous study led on Rab11A indicated a role of this GTPase in the delivery of the GPI-anchored surface antigen SAG1 at the plasma membrane of the parasite (Agop-Nersesian, C. et al., 2009). We therefore wondered whether Rab11A would play a more general role in secretory events to the parasite surface, not only for DG exocytosis and GPI anchored proteins but also for transmembrane proteins. To do so, we examined the delivery of the glucose transporter GT1 at the parasite surface after transiently expressing an HA-tagged version of the GT1 in DDmCherrycMycRab11A-expressing parasites. Similar to SAG1, GT1 localisation was systematically affected in parasites over-expressing Rab11A<sub>DN</sub> (Figure 5C) compared to parasites over-expressing Rab11A<sub>WT</sub>. GT1 was indeed detected inside the parasite cytoplasm in dispersed vesicles and did not reach the parasite plasma membrane.



Figure 5. TgRab11A influences dense granule secretion and GT1 protein delivery at early stages after invasion. (A) IFA on parasites fixed 8 hours post-invasion shows defects in the secretion of dense granule protein GRA3 in Rab11A<sub>DN</sub> parasites. (B) Western blot showing the stabilized expression of Rab11A<sub>WT</sub> and Rab11A<sub>DN</sub> in parasites 8 hours post-invasion (Left). Next, WB showing an increase in the level of GRA1 and GRA3 protein content in intracellular parasites expressing Rab11A<sub>DN</sub> upon Shield-1 induction for 8 hours post-invasion, compared to Rab11A<sub>WT</sub> induced parasites. Eno2 was used a loading control (Middle panel). The graph reflects the fold change in GRA protein expression, calculated from the intensity of the bands detected on the corresponding left WB experiment. (C) The localisation of plasma membrane anchored glucose transporter 1 (GT1) was examined by IFA in intracellular parasites 8 hours post-invasion. GT1 showed a normal peripheral localization in Rab11A<sub>WT</sub> induced parasites, in comparison to Rab11A<sub>DN</sub> induced parasites, where the protein was found retained in dispersed vesicles in the parasite cytoplasm. Scale bars = 3µm.

Taken together, our data place Rab11A as a crucial regulator of protein secretion to the parasite surface, regulating the targeting and fusion of secretory vesicles to the plasma membrane and the exocytosis of the dense granules.

### III-2.4. Rab11A dynamics during invasion

Based on the observations on GRA secretion defects in intracellular replicating parasites, we decided to probe into the secretory functions of Rab11A during host cell invasion, during which, a massive polarized secretion of GRA proteins at the apical region of the parasite is observed upon initiation of parasite vacuole formation (Dubremetz, JF., 1993).

To investigate this aspect, we first examined Rab11A localisation during the invasion process by live imaging. Using spinning disc confocal microscopy, we captured the dynamics of Rab11A during invasion using freshly egressed Rab11A<sub>WT</sub> parasites that have been pretreated with 0.6µM Shield-1 (Figure 6) for 16 hours. During entry, a movement of Rab11Apositive vesicles emerging from the Golgi area towards the apical pole was detected as the parasite propelled itself into the host cell interior (Figure 6A, 16" to 24" images). We also detected a Rab11A-signal at the base of the parasite at the end of the invasion process (21" to 35" images). By IFA, we confirmed this Rab11A localisation in parasites fixed while invading the host cell (Figure 6B). In this experiment, detection of the surface antigen SAG1, which was labeled before permeabilising the cells, allowed us to selectively mark the external part of the entering parasite, while the moving junction was labeled after mild permeabilisation with an anti-RON4 antibody (Figure 6B). Of note, upon adhesion of the parasite to the host cell (step 1), a Rab11A accumulation was observed just beneath the RON4 signal, which pinpointed the very early stage of ROP secretion and MJ formation. This observation suggests that Rab11-positive vesicles are secreted after the MJ is established, such as observed for dense granule proteins.

It is well known that a great amount of secretory activities are carried out by the micronemes and rhoptries during invasion. However, an active role of dense granule proteins in regulating the invasion process has been a popular topic of debate and has never been clearly established, mainly due to the lack of a factor that would inhibit the DG secretion process in general, thereby targeting all GRA proteins. First, based on the role of Rab11A in DG secretion that we observed in replicating parasites, we examined the localisation of the dense granule proteins GRA1 and GRA3 during the invasion process in relation to Rab11A. We were able to observe a partial co-localisation between GRA-positive vesicles and Rab11Apositive vesicles at the apical pole of invading parasites, indicating the possibility for a dense granule regulated secretion that is influenced by Rab11A (Figure 7A). In order to confirm these data obtained in fixed cells, we are currently generating a parasite line expressing GRA3-GFP (personal communication with Dr Alexandre Bougdour) to perform colocalization study with mCherryRab11A by live imaging.



**Figure 6. Rab11A dynamics during invasion.** (A) Selected frames from a movie captured during parasite invasion using spinning disk confocal microscopy. The Rab11A positive vesicles were visualized after expression of the DDmCherryRab11A<sub>WT</sub> protein upon Shield-1 induction. Between 16" and 20", while the parasite penetrates the host cell, an intense signal of Rab11A positive vesicles was detected in the Golgi area and at the apex of the parasite. As the parasite progresses further into the host cell, the vesicles appear at the basal pole of the parasite starting from 21". (B) The different stages of invasion were captured in fixed parasites and Rab11A (anti-cMyc antibodies, pseudo colored in white) localization was observed in relation to the moving junction marker RON4 (anti-RON4 antibodies, green). The detection of SAG1 (without parasite permeabilisation; in red) shows the extracellular part of the entering parasite. Arrows indicate the Rab11A positive vesicles. The cartoon represents the different events during invasion reflecting what is shown by IFA Bars:2µm

Next, to assess the role of Rab11A in DG secretion during parasite entry, we looked at parasites expressing Rab11A<sub>DN</sub>. To do so, we induced Rab11A<sub>DN</sub> parasites extracellularly for two hours with 1 $\mu$ M of Shied-1, after they were freshly egressed from host cells (Rab11A<sub>WT</sub> parasites were similarly induced with 0,6 $\mu$ M of shield-1) and allowed them to invade for two minutes at 37 °C after a centrifugation step on the HFF monolayers. Thereby, parasites were fixed during invasion and subjected to IFA. Using confocal microscopy, we observed that the polarized apical localization of GRA1 and GRA3 observed in Rab11A<sub>WT</sub> parasites was no longer detected in Rab11A<sub>DN</sub> parasites, the GRA vesicular signal being dispersed in the cytoplasm. However, this defect was always correlated to an invasion defect of these adherent parasites, prior to moving junction formation (not shown). Quantification showed a 48%

decrease in invasion in Rab11 $A_{DN}$  compared to Rab11 $A_{WT}$  induced parasites. We also monitored the ability of the Rab11 $A_{DN}$  parasites to adhere to host cells and found a 32% decrease in parasite adhesion to host cells.



#### Figure 7. Rab11A partly co-localizes with GRA1 and GRA3 during invasion.

(A) IFA followed by SIM imaging shows an apical concentration of Rab11A- and GRA1/3- positive vesicles during the invasion process (arrows). Here, step 2 of the invasion process depicted in the cartoon corresponds to the IFA panels for GRA1 and GRA3 protein detection. Of note, only a partial co-localization is observed between GRA1 / GRA3 vesicles and Rab11A. Scale bar =  $2\mu m$ . (B) IFA showing the polarised apical localization of GRA1 and GRA3 vesicles during invasion in uninduced Rab11A<sub>DN</sub> parasites, while they were found dispersed within the cytoplasm of adherent Rab11A<sub>DN</sub> induced parasites. SAG1 staining performed before permeabilisation marked the extracellular portion of the entering parasites. Scale bars =  $2\mu m$ .

These data indicate that Rab11A plays an essential role in both, parasite adhesion to host cells, likely due to the secretory defect of adhesion molecules such as SAG1, but also in the invasion process itself, observed in parasites, which had successfully adhered to host cells. Different factors could inhibit parasite invasion, such as ROP and MIC secretion that triggers moving junction formation, as well as a defect in parasite motility. Indeed, Rab11A has been

shown in mammalian cells to regulate cell motility/migration (Mammoto, Aet al., 1999; Jones M.C., et al., 2006). To address this hypothesis, we performed a motility assay where we looked for trails deposited by motile parasites on BSA coated coverslips. We found that extracellular parasites expressing Rab11A<sub>DN</sub> were severely impaired in their motility, which was evident from the drastic decrease of trails that were tracked using SAG1 staining. Leaning from these observations, we also monitored by live imaging the dynamic localization of Rab11A during motility prior to invasion (Figure 8D). An intense accumulation of Rab11A positive vesicles at the apex and base of motile parasites was observed prior host cell invasion, correlated with a dynamic movement of vesicles from the apex towards the base and vice versa.

Together, our data suggest that Rab11A-positive vesicles could regulate the transport and/or secretion of GRA proteins or other factors that are essential for the earlier stages of parasite invasion such as motility and adhesion. This hypothesis will be investigated in the near future. Importantly, the role of Rab11A in the secretion of ROP and MIC proteins is currently investigated. We are monitoring the secretion of MIC proteins upon ethanol induction by ESA (Excreted Secreted Antigens) assays (Graindorge, A. et al., 2016) and ROP-containing evacuole secretion in host cells (Mueller, C. et al., 2013) in induced extracellular Rab11A WT versus DN parasites.



**Figure 8. TgRab11A** is essential in extracellular parasites for key invasion events (A) Invasion assay was performed using freshly egressed Rab11AWT and DN parasites treated with Shield-1 for 2 hours. The graph shows the percentage of parasites that had invaded host cells after 1hour. The percentages represent the number of inside parasites over the number of total parasites: (inside + outside) and is represented as mean  $\pm$ SEM (3 independent biological replicates). (B) Adhering capacity of parasites expressing Rab11<sub>DN</sub> was calculated against that of Rab11A<sub>WT</sub> parasite (see Materials and Methods section for detailed experimental protocol). Experiments were done in technical duplicates, for 3 independent experiments. Data are expressed as mean  $\pm$ SEM. (C) The percentage of parasites showing a SAG1-positive trail deposition was counted in Rab11AWT and DN parasites following induction with Shield-1 for 2 hours. Data are presented as mean  $\pm$ SEM. (3 independent experiments) (D) A series of time frames from a movie captured on extracellular motile parasites expressing DDmcherry-Rab11A<sub>WT</sub>. The Rab11A-positive vesicular movements are indicated by arrows. Scale=2µm.

# III-2.5. Parasite cortex and glideosome components recruitment in extracellular Rab11A<sub>DN</sub> parasites

It has been previously shown that during parasite replication, Rab11A is required for the formation of a functional glideosome in the nascent daughter cells. Therefore, we envisioned that the motility and adhesion defects observed in extracellular Rab11ADN parasites could be caused by a defect in the recruitment of the glideosome components at the IMC and/or in the integrity of the parasite cortical cytoskeleton. IFA analysis revealed that extracellular induced parasites expressing Rab11A<sub>DN</sub> that have successfully adhered to the host cell, showed no obvious defects in the recruitment of GAP45 and Myosin Light Chain1 (MLC1) (Fig8A). An antibody against β-Tubulin was used to check the integrity of the cortical microtubule cytoskeleton of the parasite. In contrast to glideosome components, tubulin displayed a patchy localisation in Rab11A<sub>DN</sub> parasites, compared to a homogenous and continous signal lying the cortical area of Rab11AWT parasites. This suggested that the early defects in adhesion and motility could be due to a defect in the cortex integrity, particularly at the level of the microtubule network, but possibly also for other factors yet to be identified. Indeed, although the myosins and actin proteins are the motor proteins that have been studied the most and reported to be crucial for successful invasion, it is also known that the subpellicular microtubules are equally important. This study demonstrated that the spiral trajectory observed for tachyzoites moving in a 3D matrix requires the subpellilcular microtubule activity (Jacqueline, M.L. et al., 2014). Subpellicular microtubule cytoskeleton integrity will be investigated by electron microscopy after cytoskeleton extraction (Nichols, B.A. and Chiappino, M.L., 1987).

We also investigated parasite morphology using scanning electron microscopy (SEM), as a defect in the microtubule cortical network might have a drastic effect on the parasite shape. Rab11A<sub>DN</sub> parasites and Rab11A<sub>WT</sub> parasites were extracellularly induced with Shield-1 for 2 hours, and allowed to adhere on BSA coated coverslips before being processed for SEM. SEM analysis showed that in Rab11ADN parasites, the typical thin elongated arch shape that defines WT tachyzoites was not preserved. They exhibited a distorted morphology appearing shorter, more rounded and stumped. However, parasite polarity seems to be preserved as the conoid could be clearly detected in these parasites (arrow). These data suggest that the dynamic movement of Rab11A positive vesicles might be required to preserve parasite morphology and cortical integrity in extracellular parasites.

Α.

Rab11AWT+S < 2m	in		Rab11ADN+S 5min					
SAG1	Tubulin →	Rab11A	SAG1	Tubulin	Rab11A			
SAG1	GAP45 →	Rab11A	SAG1	GAP45 →	Rab11A			
SAG1		Rab11A	SAG1		Rab11A			

Β.



Figure 9. Cortical integrity and recruitment of glideosome components on extracellular parasites (A) IFA performed on Rab11A<sub>WT</sub> and Rab11A<sub>DN</sub> parasites fixed during the invasion process. The panel on the left shows Rab11A<sub>WT</sub> parasites fixed after 2 minutes following inoculation onto HFF monolayers. SAG1 staining (blue) prior to permeabilization reveals a faint signal in Rab11A<sub>WT</sub> parasites, which have either already invaded or in the process of invasion. In the panel on the right, extracellular Rab11A<sub>DN</sub> parasites reveal a SAG1 signal (although patchy) even after 5 minutes of inoculation onto host cells. , GAP45 and MLC1 stainings (green) allowed the detection of the glideosome components, which appear normally distributed in both the strains, while tubulin, representing the cortical cytoskeleton, showed a patchy distribution in Rab11A<sub>DN</sub> parasites. – The Rab11A signal (red) appeared as vesicles in the Rab11AWT and diffused cytosolic distribution in the Rab11ADN parasites. Scale bar =  $2\mu$ m. (B) Scanning electron micrographs reveal morphological defects in Rab11A<sub>DN</sub> parasites. The conoid (Co) is indicated by by an arrow.Scale bar =  $2\mu$ m.

### III-2.6. Rab11A interacts with the adaptor molecule TgHOOK

Our data revealed pleiotropic roles for Rab11A in parasite adhesion, motility, invasion and replication. All these functions are probably achieved via a common Rab11A-dependent secretory activity that regulates the delivery of different factors at distinct localizations of the parasite, and probably via Rab11A association to different organelle types.

In order to dissect further the molecular mechanisms regulating this Rab11A-dependent secretory activity, we aimed to fish partner molecules. To do so, we first performed immunoprecipitation (IP) experiments. However, we could'nt identify possible interesting interactors by this strategy. Hence we decided to perform a GST pull down of active Rab11A (in presence of GTP<sub>y</sub>S). Following mass spectrometric analysis, among other interesting candidates, we identified one particular molecule, a HOOK domain containing protein (TGGT1 289100), which only associated to the GTPyS bound form of Rab11A and not its GDP bound inactive form. TGGT1 289100 is a hook domain containing protein, which we address here on as TgHOOK. Bioinformatic analysis helped us to confirm the presence of a well-conserved HOOK domain, a chromosome segregation associated domains involved in regulating cell division and coiled coil domains. Hook proteins are adaptor molecules for the molecular motor dynein and play a role in vesicular trafficking by anchoring vesicles to microtubule tracks via its interaction with Rab proteins (Kramer, H. and Phistry, M., 1996; Sunio, A. et al., 1999; Maldonado-Baez, L et al, 2013). For example, in drosophila Hook was shown to be involved in anchoring of endosomes to microtubules, and Rab11 facilitates endosome-autophagosome fusion by removing HOOK from mature late endosomes and blocking its dimerization (Szatmari, M et al., 2014). We thus got interested and decided to study this protein.

To investigate the functions of this uncharacterized protein in *T. gondii*, first we generated a stable Knock-In line of this protein with a HA tag tethered to its C-terminus in order to examine its intracellular localisation. We found that TgHOOK is enriched at the apical tip of the parasite and also present in numerous cytoplasmic vesicles enriched in the 1/3 apical length of the parasite, quite similar to microneme protein pattern distribution (Figure 11A). Owing to this vesicular pattern and also its partial co-localisation with MIC2, a study carried out on novel cell cycle regulators, proposed this protein could belong to the microneme family and named it as MIC18 (Butler, C.L. et al., 2014). However, one should note that TgHOOK

does not contain any signal peptide, one of the main features of MIC proteins. However, the authors also mentioned that the protein is predicted to contain a Hook domain, known to bind to microtubules, and hence could probably function in microneme translocation in a subpellicular microtubule dependent-manner from the Golgi to the apical region of the parasite (Butler, C.L. et al., 2014).

Α.

			Total number of peptides			Total number of spectra		
Protein name	Protein accession numbers	MW (Da)	GST- GTPγS	GST- Rab11A GDP	GST- Rab11A GTPγS	GST- GTPγS	GST- Rab11A GDP	GST- Rab11A GTPγS
Ras-related protein Rab11	TGGT1_ 289680	24997	19	30	27	764	2090	1459
hypothetical protein (HOOK domain containing)	TGGT1_ 289100	78764			16			21



**Figure 10. TgHOOK localization** (A) The table shows the mass spectrometry results for the GST pull down experiment. TgHOOK was identified as a strong interactor of GTP  $\gamma$  S-bound active Rab11A, with a high amount of unique peptides. (B and C) Knock-In parasites expressing HA tagged HOOK. (B) Western blot revealed the expression of a single band with a predicted molecular weight of 79kDa, corresponding to the HA-tagged version of TgHOOK, which was not detected in the RH $\Delta$ Ku80 parental strain. Actin1 was used as a loading control. (C) IFA experiment showing that TgHOOK localizes at the apical tip of the parasite (see arrows) and also in numerous vesicles that run across the cytoplasm. Scale bar = 2 $\mu$ m. (D) Co-localization of TgHOOK with Rab11A. **The upper panel** shows

parasites in G1 phase cell division cycle where there is a partial co localization between some Rab11A and HOOK vesicles. The insets 1 and 2 indicate the regions of the vacuole that have been magnified and showed on the right side. Partial vesicular colocalization can be detected between Rab11A and TgHOOK, The **lower panel** shows a parasitophorous vacuole going through mitosis. TgHOOK appears to be stained in the pattern of a "ring" at the parasite tip corresponding to the conoid region. Colocalization studies with conoid ring markers are underway. In the zoomed out image (see inset 3), Rab11A vesicles (white) appear to follow the microtubule tracks of the parasite, with its typical helicoidal pattern. Scale = $2\mu$ m.

In order to investigate HOOK function focusing on its putative role for Rab11A-mediated secretion, we are now in the process of generating an inducible knockout for the protein. By homology to what is known for mammalian HOOK proteins, our current working hypothesis is that TgHOOK might serve as an adaptor that connect Rab11A present on secretory vesicles to the cortical microtubule network, directly or indirectly via dynein (Figure 11). In particular, we believe that this interaction might be crucial for the polarized secretion of vesicles at the apex of the parasite during motility and invasion of host cells, but might also play a role during replication. These organellar compartments might include the dense granules, secretory vesicles carrying membrane proteins or signaling factors as well as micronemes.



**Figure11 Hypothetical working model (A)** In this working model HOOK is enriched at the conoid region and associated to vesicles across the cytoplasm that would be anchored to the microtubule filaments running down from the apical polar ring beneath the conoïd. Rab11A vesicles might regulate dense granule (GRAs) transport in a HOOK dependent manner during motility and invasion (B) A molecular model depicting the motor protein dynein migrating along the microtubule filaments. HOOK serves as an adaptor between the dynein light chain and aids in cargo transport in a Rab11 dependent-manner.

## **IV- Discussion and Perspectives**

Secretion is a widely conserved function of all eukaryotic cells (Fukuda, M. 2008) and is part of the larger process of membrane trafficking. Protein cargos synthesized at the ER pass through the Golgi network for post-translational modifications and are finally delivered by vesicular transport to the plasma membrane or be released into the extracellular space (Battey, N.H. et al., 1999; Alberts, B et al., 4<sup>th</sup> edition). The final step of this process involves fusion of vesicles to the membrane in order to release the contents in a process commonly termed as exocytosis (Alberts, B et al., 4<sup>th</sup> edition). Rabs constitute one of the largest superfamily of small GTPases, which are known to play a crucial role in membrane trafficking. Rabs switch between an inactive GDP bound state and an active GTP bound state governed by their respective GAPs and GEFs. The GTP bound Rabs activate a series of processes including vesicle budding, motility, docking and fusion (detailed in the Chapter I.5).

*Toxoplasma gondii* has a limited repertoire of 12 Rab proteins (Kremer, K. et al., 2013) unlike higher eukaryotes like humans, where nearly 70 Rabs have been identified (Schwartz, S. et al., 2007). Out of the 12 Rabs in *T.gondii*, the functions of two isoforms of Rab11, namely Rab11A and Rab11B, were studied and both were shown to play important roles in the development of the Inner Membrane Complex (IMC) of the parasites. Rab11B was demonstrated to be required for IMC biogenesis by delivery of vesicles from the Golgi to the nascent IMC of the daughter cells (Agop-Nersesian et al., 2011). Rab11A on the other hand was shown to interact with Myosin Light Chain1 (MLC1) and play a role in the assembly of the glideosome at the IMC and contribute to a later stage of cytokinesis (Agop-Nersesian et al., 2009).

The authors had used the DD (Destabilization Domain) system to regulate the level of expression of the gene (Chapter II.2.1). They reported that Rab11A localizes to the endosomal-like compartment (co-localisation with the endosomal marker proM2AP) and also at the rhoptries, after the protein expression was stabilized in the presence of 1 $\mu$ M Shield-1 (Agop-Nersesian et al., 2009). The association of Rab11A with the rhoptries was also found in another study after purification of rhoptry organelles and identification of their content by mass spectrometry (Bradley, et al., 2005).

### IV-1. TgRab11A Dynamics reveals novel functions for the protein

We used the same constructs of Rab11A<sub>WT</sub> and Rab11A<sub>DN</sub> from this earlier work, throughout our study. Making use of the possibility to regulate the expression level of the protein in a ligand (Shield-1) dependent manner, we decided to explore the localization pattern of Rab11A<sub>WT</sub> in further details. We tested different concentrations of Shield-1 (0.2µM, 0.4µM, 0.6µM, 0.8µM and 1µM) to regulate the expression levels of Rab11A (Data not shown). At moderate Shield-1 induction levels (0,4 to 0.6µM), we observed a localization of Rab11A at the Golgi region and also in several cytoplasmic vesicles, particularly enriched at the polar regions of the parasite. At higher concentrations, parasites suffered from mild growth defects and at lower concentrations the signal was too weak and more diffused. The localization of Rab11A at the Golgi region prompted us to examine by IFA other standard markers of this compartment and a strong co-localization with the TGN resident AP1µ was observed (Figure1B). A similar localization pattern has been already reported for Rab11 in Drosophila photoreceptor cells (Satoh. et al, 2005), and BHK (baby hamster kidney) cells. Indeed, otherwise, Rab11 is broadly accepted to be associated with the perinuclear recycling compartment and in secretory post-Golgi vesicles (Welz, T. et al., 2014). To unambiguously confirm the localization of Rab11A, we raised a polyclonal antibody against TgRab11A in mice, using the full recombinant protein. Our results showed a dynamic localization pattern across different cell cycle stages (Figure 1D) similar to the one observed with the ectopic expression of DDRab11A<sub>WT.</sub> However, it also partially shares a similar localisation pattern with the one described for regulated ectopic expression of DDmCherrycMycRab11B in particular, during daughter cell budding (Agop-Nersesian et al., 2011). It has to be taken into account that indeed the two isoforms TgRab11A and TgRab11B share a high level of similarities at the amino acid level. Thus, the specificity of our antibody is yet to be confirmed. To do so, on one hand, we will verify the possible cross-reactivity with other Rabs transgenic by using total protein extracts from parasites expressing DDmCherrycMycRab11B<sub>WT</sub> and on the other hand, by testing our antibody against purified Rab proteins (GST-Rab11B, GST-Rab5A /C) already available in the lab.

Nevertheless, taking advantage of the mCherry tag on the DDRab11A<sub>WT</sub> construct, we were able to clearly visualize in intracellular parasites, the movements of Rab11A positive vesicles from the Golgi to the apical pole of the parasite, as well as bidirectional movements between the apical and basal poles that most of the time display a straight and fast trajectory, which follows the lateral sides of the parasite. The lateral movement of Rab11A vesicles is likely

dependent on the acto-myosin cytoskeleton activity anchored at the IMC, which covers the full length of the parasite, as the microtubule network covers only the first 1/3 portion of the parasite starting from the apical pole. Experiments using actin and microtubule depolymerising drugs as well as myosin mutants, will allow us to address this hypothesis. Importantly, we mainly visualized the movements of large and brilliant vesicles corresponding to the size of the dense granules, while fainter and smaller vesicles were detected but difficult to distinguish from the diffuse cytoplasmic signal of Rab11A. Thus, the limitation we faced with the low intensity of the mCherry-Rab11A signal probably restricts our analysis to only a sub-population of Rab11A-positive organelles. In particular, we believe that we did not monitor so far the movements of smaller secretory vesicles that might follow the microtubule tracks, as suggested in the IFA experiments presented in Figure 10.

Furthermore, we visualized Rab11A vesicle movements towards the apex during the G1 phase of the cell cycle, raising the question of the nature of the molecules transported via this Rab11A-dependent pathway. Live imaging with different organelle markers should help in elucidating this aspect. For example, two-coloured live imaging using mCherryRab11A and a marker of dense granules, or micronemes or rhoptries, should act as a first step in helping us to understand whether this secretory activity is linked to early stages of apical organelle biogenesis. We can also envision that this activity is linked to the transport of signaling factors involved in the regulation of the conoid and cytoskeleton-related functions. This notion of Rab11A carrying signaling factors is also supported by its concentration at the basal pole of dividing parasites, such as observed in mammalian cells (Wan, P. et al., 2013). The great enrichment of Rab11A-vesicles at the basal pole of dividing parasites also brings the question of the functional role of this process. Two major hypothesis could be proposed: 1this localisation favors exchanges with the vacuolar space as the IMC interrupts at this site, a process already observed for dense Granules or 2- Rab11A-vesicles transport important factors that regulates the final step of cytokinesis and in particular the activity of the basal complex and the formation of the residual body. Indeed, the residual body is in fact a wellorganised structure (see figure below for illustration), which seems to play a crucial role in preserving the synchronicity of the division events within the vacuole (Muniz-Hernandez, S. et al., 2011).



<u>Legend</u>: (adapted from Muniz-Hernandez, S. et al., 2011). Scanning electron microscopy images and transmission electron micrographs (right) showing the highly structured organization of the residual body that holds together parasites by their basal pole during intracellular replication. Bars:  $1 \mu m$ .

### IV-2. TgRab11A as a master regulator of constitutive secretion

Our knowledge from other eukaryotic cell systems, describing a role for Rab11 in both, constitutive and regulated secretion (Urbe et al., 1993; Li, et al., 2007; Sato, M. et al., 2008) prompted us to investigate the functional defects caused to the parasite by the expression of the inactive GDP-locked DDRab11A<sub>DN</sub>. In the previous study on TgRab11A, the authors had clearly demonstrated the role of Rab11 in parasite invasion, constitutive secretion of GPI anchored surface antigen SAG1 and also the assembly of the late glideosome components (GAP45 and MLC1) at the IMC, while rhoptry and microneme biogenesis was not affected (Herm-Gotz et al., 2007; Agop-Nersesian et al., 2009).

Dense granule secretion has been widely viewed as a constitutive secretion process, taking place in a continuous manner during the replication of parasites. Furthermore, analysis of DG trajectories by live imaging indicated both directed (at the parasite cortical area) as well as diffusive-like (in the cytoplasm) movements that are dependent on the activity of the actin and myosin cytoskeleton–(Heaslip, A., et al., 2016). These observations argue for a model in which, granules move randomly in the cytoplasm before being caught by the cortical actomyosin network to promote exocytosis as favorable sites, for example at the junctions between the alveolar sacs which form the IMC.

Studying Rab11A function in relation to dense granule secretion seemed to be very fitting for us due to our observations of Rab11A-vesicles movements. IFA studies had clearly pointed out that parasites expressing the GDP-locked DDRab11A<sub>DN</sub> are defective in dense granule secretion. We have tested different GRAs: GRA1, GRA2, GRA3, GRA5, GRA6, GRA16 (Fig4. Data shown only for GRA1, 3 and 16) and seen an arrest of secretion for all these proteins, suggesting a general role of Rab11A in DG exocytosis. It has to be mentioned that

the regulated expression of both DDRab11A<sub>WT</sub> and DDRab11A<sub>DN</sub> was observed as early as two hours following induction (Fig1A) and even earlier (not shown). This was also reported in the first study on TgRab11A (Herm-Gotz et al., 2007). By treating our parasites with Shield-1, we were able to observe the arrest in GRA secretion even before budding of daughter cells occurs therefore before the cytokinesis defect takes place. This indicated that the inhibition of GRA secretion is not an indirect effect of the division defect.

We also observed a drastic defect in the secretion of the transmembrane protein GT1, showing that Rab11A does also play a crucial role in delivery of membrane associated molecules, as it was shown for SAG1 (Surface antigen -1) (Agop-Nersesian, C. et al., 2009), which is anchored with a glycophosphatidyl moiety at the plasma membrane. Of note Rab11 was shown to regulate the delivery of the glucose transporter Glut3 also in neuronal cells (McClory, H et al., 2014). Therefore, our results place Rab11A as a general regulator of secretory events to the plasma membrane of the parasite. Importantly, we did not observe the accumulation of GRAs proteins or GT1 at the Golgi in Rab11A<sub>DN</sub> parasites, suggesting that Rab11A is not involved in the exit of these proteins from the TGN but rather in their transport or final step of exocytosis of carrier vesicles.

# IV-3. TgRab11A, a potential regulator of polarized secretion during motility and invasion

Having observed the drastic effects on GRA secretion during intracellular replication, we decided to narrow down our focus onto the Rab11A-mediated secretion mechanisms of GRAs proteins. The observation by live imaging of Rab11A positive vesicles concentrated at the apex of parasites during motility and invasion gave us the next clue for its putative role in polarised secretion. It is well known fact that the invasion event is a hub for massive polarized secretion. Although it is the secretion of micronemes and rhoptries during invasion that has been most widely studied, there have been very few studies that analysed and reported a regulated secretion mechanism of the GRAs (Dubremetz, J.F., 1993). We were able to clearly see that GRA proteins were accumulating at the apex during the invasion of tachyzoites into the host cell and partially colocalised with Rab11A at this position (Figure 7). However, we also observed at the onset of the invasion process, that the early accumulation of GRA proteins is seen just beneath the RON4-positive spot marking the first secretory events of ROP proteins and moving junction formation. This observation suggests that as previously
proposed, GRA secretion occurs while PV starts to form. Live imaging examining RON4 or GRA proteins together with Rab11A during the invasion process should clarify this point. However, we did quantify a defect in adhesion, motility, and invasion in Rab11A<sub>DN</sub> parasites, demonstrating a role for Rab11A in earlier events prior moving junction formation and during entry that could be independent of dense granule release into the external environment. Of note, a defect in motility would cause a defect in invasion and we favor this explanation.

Based on our live imaging observations of Rab11A accumulating at the apex of the parasite during parasite motility and adhesion prior to invasion, we could envision the possibility that crucial factors participating to these processes are exocytosed via a Rab11A-dependent pathway. To date, GRA proteins have never been identified to regulate adhesion or motility in T. gondii, however this aspect has not been clearly investigated. In particular, one hypothesis would include the Rab11A-mediated regulation of the microtubule or acto-myosin cytoskeleton dynamics during all these processes. This activity might rely on GRA positive vesicles trafficking, which are not released into the external environment, but rather regulate signaling events within the parasite, in particular at the cortical area. Interestingly, it has been very recently shown that the tubulin based cytoskeletal complex at the apex of the parasite, the conoid, plays a role in regulating motility and invasion via the activity of Myosin H, a conoid associated motor (Graindorge, A. et al., 2016). Another study on RNG2 showed that this protein links the apical polar ring to the conoid and interestingly, this marker flips its localization when the conoid protrudes (Katris, N.J.et al., 2014). The authors of the Myo H paper hypothesise that probably RNG2 could serve as the relay link between MyoH at the conoid and the glideosome associated MyoA located at the IMC, to regulate parasite motility. RNG2 knockdown also affected parasite invasion, motility and rhoptry evacuole formation and microneme secretion. Thus, it is beginning to become clear that the apical complex proteins control secretion of invasion factors, as well as motility.

In *T. gondii*, Rab11A-positive vesicles could transport some crucial regulators of the conoid activity. Notably, polarized secretion to the leading edge of motile cells or during immune synapse formation is dependent on the microtubule network as it allows fast long directional movements of vesicles from the perinuclear region, where proteins are synthetized, to the periphery. Likewise, TgRab11A-vesicles could be anchored on the microtubule tracks to rapidly reach the apical pole of the parasite from the Golgi area during the process of invasion (see also chapter IV-4 below).

On the other hand, we obtained preliminary results after performing ESA assays (Excreted and Secreted Antigens), which allow to monitor the rapid secretion of microneme proteins in extracellular parasites upon Ca2+ ionophore induction. We found a decrease in the level of secretion of some MIC proteins, and notably MIC2, in Rab11A<sub>DN</sub> parasites. This could well explain the defects in adhesion, and motility that these parasites face. However, our SEM analysis indicated that the integrity of the cortex seems to be challenged in these parasites, which would suggest additional roles (than MIC secretion) of TgRab11A for the regulation adhesion and motility.

It is thus clear that Rab11A in *T. gondii*, like in other eukaryotes, has pleiotropic effects on several functions. It is likely that Rab11A binds to different intracellular compartments, which could include dense granules, micronemes, and secretory vesicles transporting surface membrane proteins, and anchor these organelles to different transport systems (actin or microtubule dependent), all of this timely regulated at different cell division stages.

## IV-4. Towards identifying the constitutive secretory machinery in T. gondii

In mammalian cells, Rab11A was demonstrated to play a role in promoting exocytic events by stimulating the fusion of recycling vesicles to the plasma membrane via its binding to the exocyst complex subunit Exo70 (Takahashi, S et al., 2012). However, Exocyst complex components seem to not be encoded in the genome of *T.gondii* and in Apicomplexa as a whole (Klinger, C et al., 2013).

Therefore, the question remains opened on how Rab11A would promote exocytosis in *T. gondii*. Our efforts to identify partner molecules that could play a role in Rab11A-mediated secretion led us to the Hook domain containing protein, which we identified as a unique partner for active GTP-bound Rab11A. Hook has been identified as an adaptor for the molecular motor dynein (Olenick, MA et al., 2016), which rides the tubulin tracks to transport vesicles in a minus end directed manner. In *T. gondii*, the positioning of the MTOC at the apex (conoid) and also the presence of the dynein light chain1 (DLC1) at the conoid (Qureshi, et al., 2013), indicate that probably the "–"end of the microtubules are positioned at the apical pole in opposite to mammalian cells, where the MTOC is at the nuclear region with + extremities extending towards the periphery. We hypothesise that the dense granules and other vesicles may be trafficked in a microtubule dependent manner aided by Rab11A and the protein TgHOOK along the subpellicular microtubules. This hypothesis is further strengthened by our observation of Rab11A distribution on tracks, which have a pattern like

that of microtubules (Chapter III-2, Fig. 10). In contrast, recently Heaslip, A et al., showed that the trafficking of dense granules is dependent on actin and Myosin F during intracellular replication (Heaslip, A. et al., 2016). We propose that the polarized secretion of dense granule proteins at the onset of parasite invasion could be dependent on microtubules. Indeed, using SIM microscopy, we observed this interesting localization pattern of GRA4-positive vesicles during parasite entry, on what looks very much like the subpellicular microtubule tracks of the parasite. In these images, GRA4 can also be seen accumulated at the apical pole of the parasite, suggesting a directional movement of these GRA4-vesicles towards the apex (see image below). This microtubule-dependent motion would allow fast and polarized delivery of molecules at the apical pole, where most of the secretory events occur to ensure invasion, such as described during chemotactic directional motility of mammalian cells. Of note, the GRA-positive vesicles that we observed appear smaller in size than the typical dense granules, which might suggest that different secretion pathways exist for GRA proteins.



<u>Legend</u>: RH $\Delta$ Ku80 parasites were fixed during the invasion process (2 minutes post-adhesion), following the the protocol described in (Chapter II-III.4). AP $\mu$ 1-HA is labeled in green (small arrows) and allows to set the orientation of the parasite, with the Golgi localized in the anterior half of the parasite, GRA4 is detected in red. Note the accumulation of GRA4 at the apical pole during entry (arrow heads) and its distribution on what seems like the subpellicular microtubule tracks. Big arrow represents the direction of entry. Scale bar =1 $\mu$ m.

We are now in the process of dissecting the functions of the unique HOOK domain protein in *T.gondii* aiming to gain knowledge on the mechanisms regulating protein membrane secretion and DG exocytosis in the parasite, both activities being crucial for *T. gondii* survival and virulence.

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## **RESUME DETAILLE des TRAVAUX de THESE (FRANÇAIS)**

Etude du trafic vésiculaire des protéines de rhoptries et micronèmes et de la sécrétion des protéines de granules denses chez *Toxoplasma gondii* 

## I- Généralités :

*Toxoplasma gondii* est l'agent responsable de la toxoplasmose, une anthropozoonose très largement répandue dans le monde. Ce parasite peut infecter les oiseaux et les mammifères, y compris l'homme. Habituellement bénigne, la toxoplasmose peut engendrer de graves complications chez les personnes immunodéficientes (SIDA, cancer, greffe) et lors d'une primo-infection de la femme enceinte, pouvant aller jusqu'à la mort du fœtus in-utero. Les principales formes cliniques sont alors la toxoplasmose encéphalique et les rétinopathies.

Ce parasite intracellulaire obligatoire appartient au phylum des Apicomplexa, tout comme *Plasmodium*, l'agent responsable du paludisme qui tue près d'un million de personnes chaque année. *T. gondii* est une cellule hautement différenciée et polarisée. En effet, en plus des organites «classiques» retrouvés chez les autres eucaryotes (Réticulum endoplasmique (RE), Golgi, endosomes précoces, tardifs et de recyclage), le parasite possède des organites de sécrétion spécialisés, les micronèmes (MIC), les rhoptries (ROP) et les granules denses (GRA), qui caractérisent les parasites apicomplexes et qui constituent les principaux acteurs responsables de la virulence du parasite. En effet, les protéines ROP, MIC et GRA interviennent dans les processus assurant l'invasion de la cellule hôte et la formation de la vacuole parasitophore dans laquelle le parasite se multiplie de

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manière intensive. Ces protéines participent également à la modulation de la réponse immunitaire de l'hôte infecté.

L'organisation ultrastructurale polarisée du parasite, ainsi que les nombreux outils de génétique disponibles, font de T. gondii un excellent modèle de biologie cellulaire, en particulier pour l'étude de la biogenèse des organites de sécrétion et du trafic intracellulaire des protéines parasitaires. Les organites ROP et MIC sont formés à partir d'organelles précurseurs (pré-rhoptries et micronèmes immatures) lors de la réplication parasitaire. Les mécanismes impliqués dans leur formation ne sont que partiellement élucidés. Il a été montré que leur biogénèse résulte de la fusion séquentielle de vésicules issues de l'appareil de Golgi après sa réplication dans les cellules filles. Ainsi, la synthèse, la maturation et le transport des protéines de micronèmes et de rhoptries sont intimement liés à la formation de ces organites. Une étude menée dans le laboratoire a permis l'identification et la caractérisation d'un récepteur unique de type sortiline, le TgSORTLR (Sortilin-Like Receptor), impliqué dans le transport des protéines ROP et MIC du Golgi vers les compartiments endosomaux. La délétion de TgSORTLR entraîne un défaut dans la biogenèse des organites apicaux, causant une inhibition de l'invasion de la cellule hôte. En conséquence, dans un modèle d'infection chez la souris, les parasites déplétés pour la TgSORTLR deviennent avirulents. De plus, cette étude a permis l'identification de protéines s'associant avec le TgSORTLR, telles que le complexe adapteur de la clathrine AP1 et le complexe retromère, suggérant leur rôle dans le trafic intracellulaire des protéines ROP, MIC et GRA. Ces découvertes ont ouverts la voie à de nombreuses perspectives pour la recherche fondamentale permettant d'élucider les mécanismes régulant la formation et la sécrétion des protéines de rhoptries, de micronèmes et de granules denses et par conséquent, l'identification de cibles potentielles pour bloquer la virulence du parasite.

II. Rôle du complexe TgAP1 dans la régulation du trafic différentiel des protéines de rhoptries et micronèmes et dans la division parasitaire :

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Le but de notre projet était de caractériser les mécanismes moléculaires régulant le triage et le transport différentiel des protéines MIC et ROP depuis le Golgi vers leurs destinations finales distinctes lors de la réplication parasitaire. En particulier, nous avons étudié en détail le rôle du complexe adapteur de la clathrine AP1 (clathrin Adaptor Protein complex 1) dans ce phénomène. Les APs sont des complexes protéiques de 4 sous-unités ( $\alpha$ ,  $\beta$ ,  $\sigma$ ,  $\mu$ ) qui stimulent la formation de vésicules dites à « manteau de clathrine ». Le rôle du manteau de clathrine est double: d'une part, sa polymérisation provoque une déformation locale de la membrane qui permet la formation d'une vésicule de transport. D'autre part, il interagit directement ou à travers les protéines AP, avec les domaines cytoplasmiques de certains récepteurs membranaires, assurant leur concentration dans la vésicule en formation. Chez *T. gondii,* le complexe AP1 avait été identifié dans le laboratoire comme un partenaire direct de la TgSORTLR et devait par conséquent intervenir dans le triage et l'export des protéines MIC et ROP à partir du Golgi lors de la biogénèse des organites apicaux.

D'une part, nous avons pu mettre au point une technique de microscopie à fluorescence dite à super-résolution (SIM: Structured Illuminated Microscopy) afin de mieux caractériser l'organisation ultrastructurale des compartiments intracellulaires de *T. gondii.* Après avoir généré une souche parasitaire exprimant la sous-unité mu1 sous contrôle du promoteur endogène et fusionné à un tag HA, nous avons pu définir la localisation de AP1 dans le compartiment du Trans-Golgi-Network (TGN) (Figure 1). De plus, nous avons pu observer que le parasite possédait un compartiment « hybride » juxta-nucléaire composé de l'appareil de Golgi / et du compartiment endosomal-like (endosomes précoces Rab5-positifs et endosomes tardifs, Rab7-positifs) de manière similaire à ce qui est décrit chez les plantes. En effet, le traitement des parasites avec la Bréfeldine A, drogue qui disperse le compartiment Golgien (Figure 1), disperse également les compartiments endosomaux positifs pour les petites GTPases Rab5 et Rab7. De plus le compartiment TGN et ELC apparaissent physiquement associés pendant le cycle cellulaire et en particulier sont simultanément dupliqués et scindés entre les deux cellules filles.



Figure 1: Haut: SIM images montrant la localisation du complexe AP1 dans le Golgi et de vésicules cytoplasmique dispersées dans le cytoplasme du parasite. Au sein du Golgi, AP1 co-localise avec la TgSORTLR au niveau du Images de confocale démontrant la dispersion du AP1-positif taritement à la Bréfeldine A. Bars : 1 μm.

D'autre part, après avoir généré des souches parasitaires déficientes pour l'activité du complexe AP1 (souche knock-out pour la sous-unité mu1 ou mutant de surexpression inductible de la sous-unité mu1), nous avons pu démontrer que le complexe AP1 régule le trafic différentiel des protéines MIC et ROP. Ainsi, le complexe AP1 régule de manière précoce, dès leur néo-synthèse, le transport d'une sous-population de protéines MIC (MIC3 et MIC8) ainsi que des protéines de rhoptries depuis l'appareil de Golgi vers le compartiment endosomal-like (ELC). Par contre, le transport des protéines MIC2 et M2AP, qui sont localisées dans une population de micronèmes distincte de celle contenant le complexe MIC3/MIC8, n'est pas affecté après déplétion du gène codant pour APµ1.

De plus, nos résultats semblent suggérer un rôle additionnel du complexe AP1 dans les étapes plus tardives de maturation des compartiments vacuolaires contenant les protéines de rhoptries immatures (aussi positifs pour le marqueur endosomal précoce Rab5A), en organites matures ancrés au pôle apical du parasite. En effet, après sur-rexpression de APµ1, les protéines pro-ROP4 immatures sont retenues dans un compartiment positif pour le marqueur endosomal Rab5A et les rhoptries matures montrent un défaut drastique de morphologie. Elles apparaissent très atrophiées ou correspondant à des compartiments vacuolaires, partiellement co-localisant avec les protéines immatures Pro-ROP. Il est à noter que, dans cette souche parasitaire, la morphologie du compartiment ELC-like semble modifiée, suggérant un rôle de AP1 plus général dans la régulation de la dynamique membranaire du compartiment ELC-like Rab5A-positif.

D'autre part, par des expériences d'immuno-précipitation suivie d'analyse en spectrométrie de masse, nous avons pu identifier un nouveau partenaire de AP1, l'unique protéine Epsine de ce parasite, que nous avons nommée Epsin-like-Protein (EpsL), qui n'avait pas encore été décrite chez *T. gondii.* Chez les mammifères, les protéines de la famille epsine, lient les complexes APs et les phospho-inositols, et stimulent la courbure de la membrane lipidique afin de promouvoir la formation des vésicules à clathrine. Par des expériences de GST-pull-down, nous avons pu démonter que AP1 interagit avec EpsL via le domaine exposé « ear » de la sous-unité gamma1 et que la protéine co-localise au niveau du Trans-Golgi-Network avec AP1. De plus, de manière similaire à AP1, nos résultats préliminaires suggèrent que la protéine EpsL régule le trafic intracellulaire des protéines ROP et MIC mais n'est pas impliquée dans le processus de division parasitaire.

Finalement, notre étude a permis de révéler un rôle original du complexe AP1 dans la régulation du processus de division cellulaire du parasite. Le complexe AP1 semble crucial pour l'étape ultime de ségrégation des cellules filles. En effet, les étapes précoces de duplication du centromère et du centrosome, ainsi que le processus de budding des cellules filles, ne sont pas affectées. Nous proposons maintenant de caractériser plus en détails les mécanismes par lesquels, AP1 régulerait la division cellulaire. Deux hypothèses sont en cours d'étude : 1) AP1 régulerait la sécrétion à la membrane plasmique de vésicules provenant du Golgi permettant l'apport de lipides et la scission des cellules filles en formation ; 2) AP1 régulerait l'activité du complexe basal, incluant en particulier

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la protéine MORN1, permettant la constriction finale du cytosquelette des nouvelles cellules filles et donc leur séparation.

Cette étude a donc permis de révéler le complexe AP1 comme un facteur clé régulant plusieurs activités essentielles à la survie du parasite: la biogénèse des organites apicaux rhoptries et micronèmes et la division parasitaire (Figure 2). Par conséquent, nos résultats nous encouragent à continuer la caractérisation du complexe AP1 ou de ses partenaires d'interaction afin d'identifier des cibles moléculaires clés permettant d'inhiber la virulence de *T. gondii*.

Ces résultats ont fait l'objet de l'écriture d'un manuscrit qui a été soumis pour publication au journal *Plos Pathogens* et qui est actuellement en cours de révision avant son acceptation définitive.



**Figure 2** : <u>Gauche</u> : Modèle moléculaire du complexe fonctionnel de transport impliquant AP1. AP1 interagit avec le récepteur TgSORTLR par son domaine de reconnaissance du motif DiLeucine présent dans la queue cytoplasmique de TgSORTLR. AP1 recrute la clathrine par les domaines « Hinge » des sous-unités beta et gamma, et la protéine TgEpsL par le domaine « ear » de la sous-unité gamma. Ce

complexe stimule la formation de la vésicule à clathrine et le transport des cargos, à savoir les protéines de rhoptries et micronèmes depuis le TGN. **Droite** : Schéma résumant les différentes voies de trafic intravésiculaire régulé par AP1. AP1 active le transport des protéines MIC3 et MIC8 depuis le TGN vers le compartiment endosomal-like (ELC) mais n'est pas impliqué dans le transport des protéines M2AP et MIC2. De plus, AP1 régule le transport des protéines de rhoptries depuis le TGN vers le compartiment ELC mais participe également aux étapes plus tardives de maturation des compartiments immatures pré-ROP en rhoptries matures ancrées au pôle apical du parasite. Finalement AP1 serait impliqué dans l'étape finale de ségrégation des nouvelles cellules filles lors de la division cellulaire (anneau vert).

## III. Caractérisation des mécanismes moléculaires régulant la sécrétion Rab11A-dépendente des protéines de granules denses.

Lors de notre étude sur AP1, nous nous sommes intéressés au rôle éventuel de ce complexe dans l'activité de sécrétion à la membrane plasmique, qui semble impliqué dans les étapes finales de division du parasite. L'étude de cet aspect du trafic intracellulaire nous permettait de manière plus générale d'identifier certains mécanismes régulant l'activité de sécrétion constitutive, encore inexplorée chez *T. gondii* et pourtant essentielle à sa survie et virulence. En effet, ces voies assurent le transport des protéines transmembranaires de surface requises pour de nombreux processus tels que, l'import des nutriments de la cellule hôte, l'adhésion et la motilité parasitaire. D'autre part, cette activité est impliquée dans la sécrétion des granules denses (GRA). Les protéines GRA sont exocytées dans l'espace vacuolaire pour former un réseau membranaire de tubules qui participent à l'organisation intravacuolaire des parasites et à favoriser l'échange entre les parasites et la cellule hôte. De plus, les protéines GRA représentent d'importants facteurs de virulence sécrétés au-delà de la VP dans le cytoplasme et le noyau de la cellule hôte et qui modulent la réponse immunitaire et métabolique de l'hôte infecté.

Par des expériences de co-immunoprécipitation (Co-IP), nous avons identifié la petite GTPase Rab11A comme un partenaire potentiel de AP1. De manière importante, cette petite GTPase est la seule décrite chez *T. gondii* qui participe à une activité de sécrétion constitutive, en régulant le transport de l'antigène SAG1 à la surface du parasite. Ces résultats présentaient donc Rab11A comme un candidat très intéressant pour la régulation de l'activité de sécrétion constitutive chez *T. gondii*.

Par des expériences de marquage en immunofluorescence, nous avons observé que Rab11A se localise au niveau du TGN mais aussi sur des vésicules cytoplasmiques particulièrement enrichies au pôles basal et apical du parasite (Figure 3) lors de la réplication intracellulaire mais aussi durant les étapes précoces d'invasion de la cellule hôte, en accord avec son rôle potentiel dans une activité de sécrétion.



**Figure 3**. <u>Gauche</u> : Images de microscopie confocale montrant la localisation de Rab11A au sein du compartiment TGN, démontré par sa co-localisation avec le complexe AP1 (vert). Les études de co-localisation avec le marqueur endosomal Rab5 (vert) démontre une localisation de Rab11A antérieure au compartiment ELC dans la voie de sécrétion comme précédemment observé pour AP1. Bar : <u>Droite</u> : Pendant l'invasion de la cellule hôte, Rab11A (blanc) se concentre dans des vésicules cytoplasmiques enrichies aux pôles basal et apical du parasite. Le marquage RON4 (vert) illustre la jonction mobile et le marquage SAG1 (rouge), antigène majeur de surface du parasite, illustre la portion externe à la cellule hôte, de la membrane du parasite en train d'envahir.

En utilisant un système de sur-expression inductible de la protéine Rab11A sous une forme mutée rendant la protéine non fonctionnelle, nous sommes parvenus à inhiber l'activité de la protéine endogène par un mécanisme dit de "dominant-négatif". L'étude de ces parasites nous a permis de mettre en évidence un nouveau rôle de Rab11A dans la sécrétion des granules denses au sein de l'espace vacuolaire mais aussi du transport du récepteur transmembranaire au glucoce (GT1), pendant la réplication intra-vacuolaire. De plus, en utilisant des expériences de vidéo-microscopie à fluorescence en temps réel et de microscopie à super-résolution, nous avons pu observer une localisation polarisée au pôle apical du parasite, de vésicules Rab11A-positive lors de la motilité cellulaire (Figure 3). Ces vésicules co-localisent partiellement avec les granules denses, lors de l'invasion de la cellule hôte. De plus, les parasites inhibés dans l'activité de Rab11A montrent un défaut de motilité et d'invasion de la cellule hôte, suggérant que cette activité de sécrétion Rab11Adépendente joue un rôle crucial dans la régulation de ces activités parasitaires. De plus, ces parasites semblent présenter une morphologie altérée suggérant une perturbation des structures corticales membranaires ou du cytosquelette (Figure 4).



**Figure 3** : Série d'images extraites d'un film pris en microscopie confocale montrant la localisation de vésicules Rab11A-positive enrichies aux pôles apical et basal dans un parasite extracellulaire en mouvement. Bar : 5µm.

Une hypothèse de travail que nous considérons est que l'exocytose polarisée des granules denses régulée par Rab11A dans les parasites extracellulaires en mouvement permettrait de libérer des facteurs de signalisation essentiels à la régulation de la dynamique du cytosquelette de microtubules. En particulier, ces facteurs pourraient réguler l'activité du conoïde, structure apicale qui maintient l'organisation et la dynamique du cytosquelette parasitaire. D'autre part, Rab11A pourrait réguler l'exocytose de vésicules contenant des protéines d'adhésion aux cellules hôtes, tels que les protéines de micronèmes, qui sont essentielles à la motilité et l'invasion du parasite.



**Figure 4**. Images de microscopie électronique montrant un parasite extracellulaire contrôle et un parasite extracellulaire induit pour la sur-expression de la forme mutée inactive de Rab11A. La flèche indique la position du conoïde, et donc le pôle antérieur du parasite. Bar : 2µm.

Afin de mieux caractériser ce processus au niveau moléculaire, des expériences de GST-pull down, suivies de spectrométrie de masse ont été réalisées et ont permis d'identifier de nouveaux partenaires d'interaction de la forme activée de Rab11A. En particulier, nous nous sommes intéressés à une protéine hypothétique jamais caractérisée auparavant chez *T*.

gondii, qui présente de fortes homologies avec les protéines HOOK des cellules de mammifères. Les protéines HOOK sont connues comme des adapteurs qui ancrent les vésicules sur les microtubules via leur interaction avec les moteurs moléculaires de type dynéine, afin de stimuler leur transport directionnel. De plus, il a été montré chez les mammifères une interaction directe entre les petites GTPAses Rab 5 et Rab11 et la protéine HOOK1. Cette protéine représente donc une cible intéressante pour bloquer l'activité de sécrétion chez T. gondii. Afin de caractériser la protéine TgHOOK et son rôle potentiel dans la régulation de l'exocytose polarisée des GRA ou des micronèmes d'une manière Rab11Adépendante, nous avons généré des parasites knock-In exprimant la protéine avec une étiquette HA afin d'étudier sa localisation au sein du parasite. Nous avons pu observer que TgHOOK est localisée dans de nombreuses vésicules cytoplasmiques et se concentre à l'apex de la cellule au niveau du conoïde, en accord avec la localisation de Rab11A. Nous venons d'obtenir des parasites délétés pour TgHOOK par une approche basée sur l'utilisation de la technique CrisPR/Cas9, récemment établie au laboratoire. Les études d'une part de biochimie, visant à identifier les domaines d'interaction directe entre Rab11A and TgHOOK, et d'autre part, de caractérisation phénotypique du mutant KO pour TgHOOK, sont en cours de réalisation. Notre but est de démontrer que la protéine TgHOOK ancre les granules denses ou d'autres compartiments Rab11A-positifs sur les rails de microtubules via son interaction avec la dynéine d'une part, et Rab11A d'autre part (Figure 5). Ces interactions seraient essentielles pour réguler la sécrétion polarisée au pôle apical, des protéines GRA ou d'autres facteurs, assurant ainsi la motilité du parasite et l'invasion de la cellule hôte. Nous prévoyons la fin de ce projet, suivi de l'envoi du manuscrit pour publication très prochainement.

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**Figure 5.** Modèle de transport des vésicules Rab11A-positive par l'activité de la protéine TgHOOK. (A) Dans ce modèle hypothétique, la protéine TgHOOK qui est enrichie au conoide et présente sur des vésicules intracellulaires, permettrait l'ancrage des vésicules Rab11A-positive sur le cytosquelette de microtubules via son interaction avec la chaîne légère du moteur moléculaire dynéine (DLC1). Ceci permettrait la sécrétion polarisée de vésicules vers le pole apical du parasite, en particulier durant la motilité cellulaire et l'entrée du parasite. Ces vésicules Rab11A-positive pourraient correspondre aux granules denses, aux micronèmes ou à des vésicules sécrétoires contenant des facteurs de signalisation régulant l'activité du conoide et du cytosquelette. (B) Modèle moléculaire illustrant le complexe de transport formé par la vésicule Rab11A se liant à la protéine TgHOOK, qui recrute à son tour la dynéine pour permettre l'ancrage aux microtubules et le transport vers l'extrémité – de ces derniers.

## **CONCLUSION FINALE**

Notre étude du rôle du complexe AP1 et de Rab11A chez T. gondii nous a permis de révéler

différentes fonctions pour ces facteurs parasitaires durant l'entrée et la réplication parasitaire :

Un rôle de AP1 dans le trafic <u>différentiel</u> des protéines de micronèmes et de rhoptries depuis
le TGN et dans le processus plus tardif de maturation des rhoptries matures.

Un rôle de AP1 dans l'organisation structurale de sous-domaines fonctionnels de triage au niveau du TGN a été déjà suggéré dans les cellules de mammifères. Ce rôle pourrait être conservé chez *T. gondii* afin d'assurer le triage différentiel des protéines ROP et MIC localement au niveau du TGN vers des destinations distinctes. Le rôle de AP1 dans la maturation des rhoptries pourrait faire appel à une activité de AP1 dans la régulation de la dynamique membranaire du compartiment endosomal Rab5. Une autre hypothèse à envisager, serait un mécanisme AP1-dépendent, d'adressage ou de recyclage de facteurs essentiels rendant les compartiments rhoptries en cours de maturation compétents à leur fusion et ancrage au pôle apical.

- Un rôle de AP1 dans la division cellulaire, en particulier pour l'étape finale de ségrégation des cellules filles. Nos futures études nous permettront de comprendre si ce rôle de AP1 est lié à une activité de transport et fusion de vésicules provenant du Golgi au point de scission des cellules filles ou à une activité de régulation du complexe basal régulant l'étape finale de cytokinèse.
- Un rôle de Rab11A dans la sécrétion constitutive des granules denses et de protéines transmembranaires durant la réplication parasitaire mais aussi durant la motilité et l'entrée dans la cellule hôte. De plus, nos études ont permis de suggérer une relation fonctionnelle entre Rab11A et la protéine adaptrice TgHOOK pour cette activité.

Nous envisageons qu'il puisse exister un mécanisme unique d'exocytose Rab11A-dépendant impliqué dans toutes les activités parasitaires régulées par Rab11A : adhésion, motilité, entrée et réplication. Cette activité devrait donc partager des effecteurs moléculaires communs, notamment pour des protéines adaptrices permettant l'ancrage des vésicules aux

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moteurs moléculaires de type dynéine, kinésine et myosines ; des protéines SNARES ou des homologues éventuels du complexe « exocyste » décrit dans les cellules de mammifères.

Ainsi les travaux réalisés au cours de cette thèse auront permis de révéler des aspects nouveaux de la régulation du trafic intracellulaire chez *Toxoplasma gondii*. Ces mécanismes pourraient être conservés chez les autres parasites Apicomplexes, notamment, chez *Plasmodium*, l'agent causal de la Malaria. En particulier, le rôle de Rab11A dans la sécrétion des protéines membranaires et l'exocytose de facteurs de virulence devraient être explorés en détails pour son importance dans la survie et la pathogénicité du parasite. Par exemple, la caractérisation des mécanismes de sécrétion des antigènes majeurs que sont les protéines de granules denses, pourraient avoir une incidence sur la recherche en immunologie mais aussi sur l'impact pour la mise en place de la Toxoplasmose chronique et le développement des kystes cérébraux.

## Key Words

Toxoplasma gondii Apicomplexa Parasite Anterograde trafficking AP1 Epsin Like protein Rab11A Hook Microneme Rhoptries Dense granules Constitutive secretion