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Golgi and centrosome cycles in *Toxoplasma gondii*

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1   Introduction

1.1   *Toxoplasma gondii*

Toxoplasma gondii is an obligate intracellular protozoan parasite. It is a bow-shaped (*toxon*: ancient Greek for "bow", *plasmein*: ancient Greek for "to form / shape") highly polarized unicellular organism of about 2x8 micrometers in size. By electron microscopy it has been established that *Toxoplasma gondii* has a single nucleus, mitochondrion, plastid, interconnected ER network, and Golgi apparatus as well as an apically clustered complex of secretory organelles. [26]

The nucleus is centrally located, essentially bisecting the organism. The ER, although distributed throughout the cell, is concentrated posterior to the nucleus, and is so reduced that the nuclear envelope itself provides a substantial fraction of the ER volume. Thinly coated vesicles bud from the anterior end of the nucleus / ER, destined for the closely juxtaposed Golgi stack. [19; 26]

Toxoplasma gondii is a member of the phylum Sporozoa (Apicomplexa). This group of endoparasites shares certain common characteristics, i.e. a complex of organelles in the parasite's apical region that serve important functions in the secretory pathway and during host cell invasion. These unique polarized secretory organelles are termed micronemes, rhoptries and dense granules. Sequential secretion from these organelles is essential for host cell invasion and the formation of an intracellular parasitophorous vacuole enveloping the parasite. [12; 36; 67; 69]

1.1.1 The parasite life cycle

The parasite has a complex life cycle. Upon entry into the definitive host (all animals from the *Felidae* family, but primarily domestic cats) by oral uptake and invasion of the intestinal epithelium, parasites replicate in an asexual fashion (enteroepithelial phase). Parasites at that stage are called merozoites. Replication can occur for a couple of generations until, by the process of gamogonia, sexually differentiated stages and oocysts are produced. This can only occur in the intestinal epithelium of the definitive host.

After a short interval of a week, the newly formed oocysts can be secreted with the feces (external phase). At room temperature they sporulate within two to four days, to gain infectivity. Under suitable conditions they can remain infective for up to five years. Standard disinfectants cannot kill the parasites, but at temperatures above 60°C they die within minutes.

After uptake of a sporulated oocyst by an intermediate host (nearly all warm-blooded vertebrates and birds), sporozoites invade the organism through the intestinal epithelium and spread haematogenicly and lymphogenicly (extraintestinal phase). They can infect virtually any nucleated cell of the body, but there seems to be a preference for muscle, nerve tissue and the reticuloendothelial system. [13; 14; 18; 25; 43; 75]

In host cells the parasites develop into endozoites, also called tachyzoites (*tachys*: ancient Greek for "fast", indicating the fast replication rate), an asexual stage of differentiation. Tachyzoites replicate within a parasitophorous vacuole, whose membrane is derived from the host cell plasma membrane. They duplicate by a mechanism called endodyogeny. Two daughter parasites are formed simultaneously within each mother parasite, sharing her organelles and membrane material. [22; 25; 26; 61] In this manner the duplicating tachyzoites form rosettes within the vacuole through additional simultaneous replication events. Under the microscope, they resemble sunflowers, consisting of a number of parasites that is always a power of two (2,4,8,16,…), see Figure 1. When rosettes reach a sufficient size, parasites lyse out of the host cells and are ready to infect other cells in their vicinity.
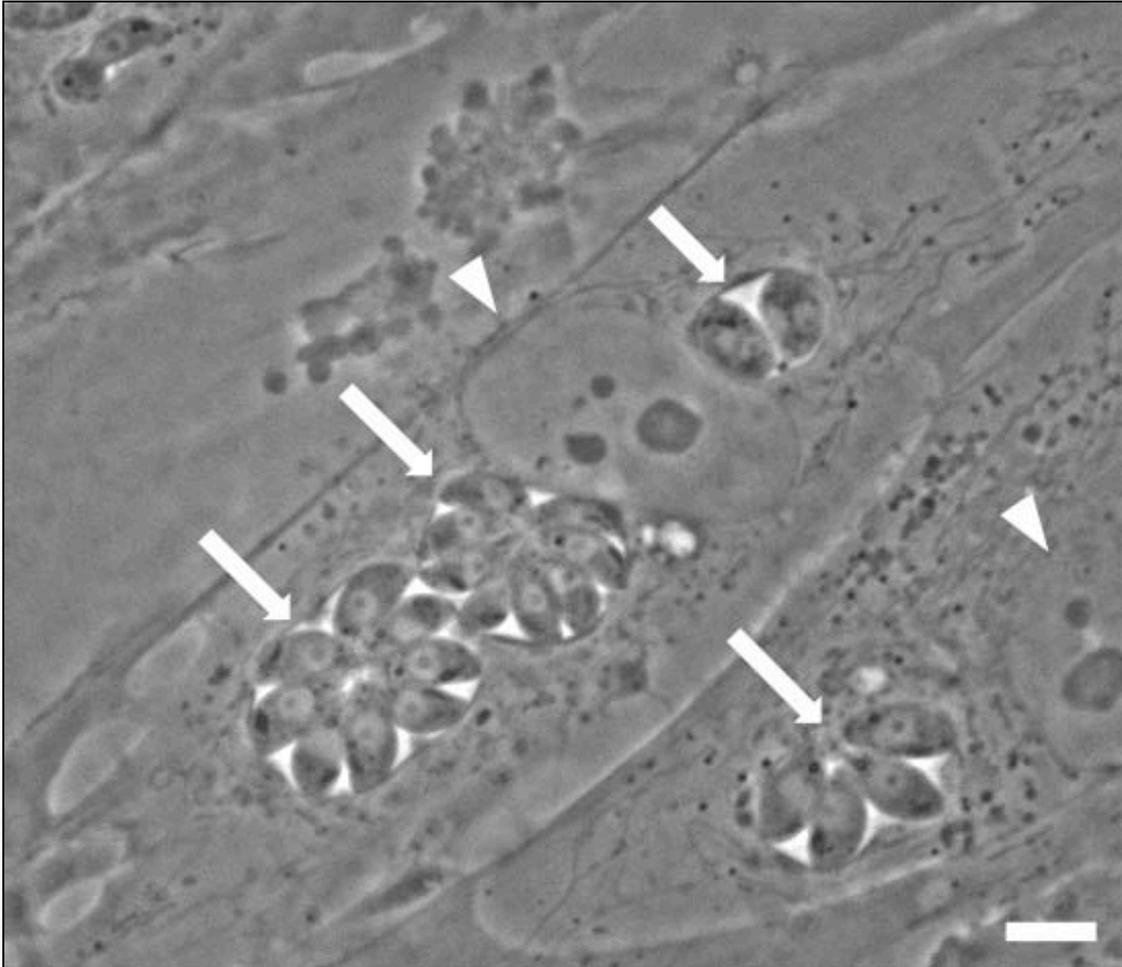
Figure 1 – *Toxoplasma gondii* tachyzoites. Phase microscopy image of vacuoles containing *Toxoplasma gondii* parasites (arrows) in HFF host cells (nuclei marked with arrowheads). Scale bar is 5µm.

If the infection enters a chronic course, parasites within tissue cysts shift their metabolism towards a slower and longer-lasting mode. This persistant form of parasites is called cystozoites or also bradyzoites (*bradys*: ancient Greek for "slow", indicating the slow replication rate). An average cyst can be up to 150 micrometers in diameter and can hold up to a couple of thousand bradyzoites. These parasites can remain in the host organism throughout its life. [43] If the host becomes the victim of a predator, parasitic cysts can be passed on, and a new cycle begins. In cats (definitive hosts) parasites can also develop into oocysts.

Infection of humans can mostly occur in two ways: either by eating raw or undercooked meat of infected animals or by orofecal uptake of cysts from cat feces. [18; 43; 75]

Although rare, possible infection from donor organs or contaminated blood or bone marrow has to be kept in mind. [24; 43; 68] Of special clinical importance, infection of an unborn can occur in a first-time infected mother, who cannot provide a sufficient immune response. [18; 38; 43; 57; 59; 73]

1.1.2 Immunity

Toxoplasma gondii triggers various immune reactions. They lead to the formation of antibodies as well as a cellular immune response and a limitation of further spreading. Immunity is primarily mediated through cellular mechanisms (T-cell system) and the production of gamma-interferon. By forming cysts the parasites can successfully hide from the immune system. This way they can persist throughtout the life of the host, even in immunocompetent individuals. Immunity is maintained as a result of the continuous presentation of small amounts of antigens, e.g. when a cyst lyses. [11; 31]

1.1.3 Epidemiology and prevention

Toxoplasma gondii is spread worldwide. The seroprevalence is estimated to be between 10 and 80 percent. In Germany, it ranges between 26-54%, in the United States between 10-30%. Prevalence increases with age. Women carry antibodies more often than comparable men. [6; 16; 18; 43; 57; 59]

In order to reduce the chance of acquiring an infection from meat (esp. pork and lamb), it should be well cooked or fried. Risk can also be reduced by limiting exposure to cats and by the use of high hygiene standards. This includes washing hands before and wearing gloves while preparing food. Sporulation of the oocysts, and therefore infectivity, requires at least two days after the oocysts have been secreted with the feces. Therefore daily cleaning and careful hygiene of cat litter is a powerful means by which to reduce the risk. [10; 18; 43]

1.1.4 Clinical aspects of *Toxoplasma gondii* infection

Most primary infections are subclinical. The patient does not even notice the infection and parasites persist asymptomatically in cysts, mostly in muscle and nerve tissue. In up to ten percent of the patients the infection causes symptoms. The disease is called toxoplasmosis. Common findings are lymphadenitis (mostly nuchal and cervical), flu-

like symptoms including fever, headache, myalgia and tiredness, as well as uveitis and hepatitis. [43]

People with a compromised immune system are at a much higher risk to develop toxoplasmosis. These patients also develop more severe symptoms than healthy individuals. Most dangerous are infections that spread to the brain and lead to multiple coagulation necroses, hemorrhages and brain edema. Other severe or even life-limiting conditions include pneumonia, hepatosplenomegalia, myocarditis and chorioretinitis. [18; 24; 43]

If a woman gets infected with *Toxoplasma gondii* for the first time during pregnancy, the unborn is at risk of a transplacental infection. In seropositive women there is only a very low risk. Congenital infection can lead to severe malformations and even stillbirth. The earlier the infection occurs during the pregnancy, the more severe the symptoms. On the other hand, the likeliness of infection is lower in early stages and increases during the course of pregnancy. Only about one percent of the surviving children show the classic clinical triad of hydrocephalus, chorioretinitis, and intracerebral calcifications as a result of the encephalitis. Up to ten percent show non-specific symptoms like fever, splenomegalia, hepatomegalia, jaundice, lymphadenitis, and anemia. The rest do not show any symptoms at birth, but may develop symptoms in the following months or years, most commonly chorioretinitis and mental retardation. [18; 38; 43; 57; 59; 73]

Only clinically apparent infections, toxoplasmosis of immunocompromised patients and (possible) first-time infections among pregnant women require pharmaceutical treatment. The standard therapy consists of a combination of pyrimethamine and sulfadiazine (calciumfolinat is added for protection from myelotoxic side effects) over a period of four to six weeks. Sulfadiazine may be replaced by clindamycin in AIDS patients. [42; 43]

1.2   Golgi apparatus

In 1897 Camillo Golgi discovered a novel intracellular structure. He reported on his findings to the Medico-Surgical Society of Pavia in April 1898, describing it as an

"internal reticular apparatus". [17] In honor of Camillo Golgi this organelle is referred to as the Golgi apparatus or simply the Golgi.

It is an organelle found in eukaryotic cells. It typically consists of a series of flattened cisternal membranes closely apposed and aligned in parallel to form a stack. The Golgi apparatus of mammalian cells lies close to the nucleus and the centrioles. Small Golgi stacks are connected to link equivalent cisternae and to form a compact ribbon with a convex-concave shape. Ultrastructural and biochemical studies have shown that the Golgi apparatus can be subdivided into several functional compartments (cis, medial and trans-Golgi). This allows certain reactions and modifications to occur in optimized and discrete microenvironments and it gives the Golgi structural as well as functional polarity. [74] The Golgi stack is bound on either side by tubulovesicular networks: the cis-Golgi network, facing towards the membranes of the ER, and the trans-Golgi network (TGN), facing towards the cell surface.

1.2.1 Golgi functions

The Golgi apparatus occupies a central position in the classical secretory pathway. The cis-Golgi network receives the entire biosynthetic output from the ER, whereas the trans-Golgi network sorts completed, posttranslationally modified products onto their final destination. [39; 65] Resident enzyme complexes in the intraluminal milieu of Golgi cisternae conjugate secretory cargo with elaborate and highly diverse patterns of glycans. [21] The complexity of glycosylation conferred by Golgi glycosyltransferases creates very distinct and specific patterns. It gives the glycocalix its unique character, which creates the cell's identity, and it facilitates functions of particular importance for the adaptive and innate immune response. [55]

At the center of the secretory pathway, the Golgi is dependent on ordered membrane-flux, which in turn requires proper fusion and budding of membranes. Vesicles carrying newly synthesized proteins from the ER fuse on the cis-side to undergo peripheral modifications in the lumen of the stacks of the Golgi apparatus, where they are passed on from one compartment to the other. After successful modification vesicles bud off from the trans-side, serving multiple purposes.

One class of vesicles, so-called transport vesicles, carry constitutively expressed proteins, such as albumin in hepatocytes, to the apical plasma membrane. Others,

termed secretory vesicles, transport proteins from the trans-Golgi towards the cell surface, to be released only upon an external or internal stimulus. Some vesicles enter the endocytic system to form primary lysosomes and after a maturation process secondary lysosomes. Via the salvage pathway, membranes coming from the endoplasmic reticulum, which are labeled with the KDEL amino acid sequence, are sent back to the ER. Contrary to the other vesicle populations, these bud off from the cis-Golgi side. Their purpose is to provide the endoplasmic reticulum with membrane material for further transport vesicles to the Golgi apparatus and other destinations.

1.2.2 Golgi inheritance

The formation of two daughter cells in the process of cell division requires proper organellar inheritance, to provide each of the daughters with a sufficient and functioning set. In a first step the organelle grows in size in preparation for cell division. Then it needs to be equally distributed among the progeny. To this end, there are two different strategies, which can be used exclusively or combined depending on the necessities. Ordered partitioning (e.g. chromosomes) in most cases utilizes the mitotic spindle and the adjacent microtubular network. Random or stochastic partitioning (e.g. mitochondria) provides each of the daughters with approximately the same amount of organelles. This is possible because the organelle is present in multiple copies and distributed evenly throughout the cytoplasm. [65; 77]

Biogenesis and inheritance of the Golgi apparatus is a very controversial field. During interphase the Golgi apparatus needs to duplicate. The mechanism and regulating factors remain unknown. At the beginning of mitosis in prophase, the Golgi ribbon located near the nucleus falls apart into many stacks. This process seems to be related to a reorganization of the microtubular network, as experimental disruption of microtubules with nocodazole shows similar effects. [9]

The transition from prometaphase to anaphase and the inheritance of the Golgi apparatus is controversial. Two models have been proposed. The first regards the Golgi apparatus as an autonomous organelle and suggests that mitotic clusters fragment and sort into daughter cells, the second proposes that the Golgi merges with the ER for inheritance and therefore should be regarded as an extension of it. [34; 65]

According to the first model the perinuclear stacks formed in prophase further fragment into tubulovesicular clusters, called mitotic Golgi clusters (MGC). [41] This fragmentation can be understood as a result of mitotic inhibition of Golgi membrane fusion events. It coincides with increased levels of CDK1 kinase activity, that also trigger nuclear envelope disassembly and microtubule rearrangements. [48] The equilibrium is thereby shifted from stacked Golgi cisternae towards clusters of vesicles. [65; 76] MGCs orient radially around the nascent mitotic spindle asters. At the beginning of metaphase, they divide into two subpopulations. One remains with the spindle poles, the other is dispersed throughout the cytoplasm by interaction with astral microtubules. To balance the amount of Golgi membrane on either side, MGCs can equilibrate between spindle poles until anaphase. [27; 62] With the sudden decrease in CDK1 kinase activity at telophase the pre-existing MGCs are used as a template to re-form stacks, which grow. Once they have reached a sufficient size the stacks coalesce in the juxtanuclear region to reform the Golgi ribbon. [63]

The second model claims that the Golgi apparatus is in dynamic equilibrium with the ER. [32; 78] During interphase this equilibrium can be perturbed by Brefeldin A (BFA) treatment or experimental ER exit block. Both cause Golgi enzymes to be redistributed to the ER [33; 58]. The mitotic ER exit block [15] is suggested to lead to a fast retrograde flow of Golgi residents to the ER. MGCs are supposed to be the transport form. Inheritance occurs stochastically along with the ER, before Golgi material is released again with the end of mitotic ER exit block at telophase to form a new Golgi apparatus *de novo*. [78]

1.2.2.1   Golgi reassembly Stacking Proteins (GRASPs)

Golgi reassembly stacking proteins (GRASPs) are members of a family of Golgi matrix proteins, required for the stacking of Golgi cisternae during reassembly. Two GRASPs have so far been described: GRASP65 [3; 4] and GRASP55. [64; 66] Both of them serve essential functions in the reassembly process by linking different components of the stacking machinery. They contain CDK1 phosphorylation sites and are phosphorylated during mitosis, which may influence the interactions that hold cisternae together. [65] GRASP55 is localized to the medial Golgi cisternae and has therefore been implicated in stacking mainly medial cisternae. [52] There is no clear *Toxoplasma gondii* homologue to it.

1.2.3 Golgi biogenesis in *Toxoplasma gondii*

Golgi biogenesis in *Toxoplasma gondii* occurs as an ordered series of events. Prior to mitosis the existing Golgi grows laterally by extension. When a critical size is reached, the Golgi undergoes medial fission, which yields two copies. These copies are divided once more to temporally form four Golgi copies, before they fuse to form two copies, one of which is incorporated into each daughter parasite. [51]

1.3   Centrosomes

1.3.1 Function and ultrastructure

Centrioles are cylindrical structures found close to the nucleus at the center of the microtubular network. Centrioles act as seeds to recruit microtubule-nucleating material, referred to as pericentriolar material, to give rise to a centrosome. Centrioles can also act as structural templates to initiate the assembly of cilia and flagella, and are then referred to as basal bodies. [37]

In interphase cells, two centrioles are surrounded by a diffuse mass of proteins, the pericentriolar mass, to form the centrosome. It is typically located close to the nucleus and serves as the origin of cytoplasmic microtubules. This intimate relation to the microtubular network has brought the centrosome yet another name: **m**icro**t**ubule **o**rganizing **c**entre (MTOC).

During cell division, the primary function of the centriole is to recruit microtubule-nucleating material and to concentrate it at a discrete location. This is necessary to subsequently form the astral mitotic spindle. Since astral microtubules play an important role in positioning the spindle during cytokinesis, the removal or ablation of centrioles from mammalian cells lead to errors in cytokinesis. [28; 37]

Each centriole consists of a nine-fold symmetrical array of triplet microtubules, called blades. The distal end contains the plus-ends of the microtubules, and coordinates the assembly of cilia and flagella, when centrioles turn into basal bodies. The proximal end of the centriole contains the 'cartwheel', a set of nine spokes connected to a central axis. [37]

1.3.2 Centrin

One component of the centriole is a protein called centrin, which is located mostly at the distal end of the centriole blade. [37; 49] The yeast homolog of centrin, Cdc31, is responsible for forming a half-bridge structure that gives rise to a new spindle pole body, suggesting that centrin might play a similar role in centriole duplication. [1] This hypothesis has been supported by experiments interfering with centrin function [29; 40] and observations that the phosphorylation of centrin triggers the separation of the mother centriole pair before centrosome duplication. [35]

Centrin has been used as a target for immunolabeling of centrioles in mammalian cells as well as in *Toxoplasma gondii* parasites. [56; 71]

1.3.3 Centrosome duplication

New centrioles form both adjacent and at right angles to pre-existing centrioles. The daughter centriole does not incorporate any part of the mother centriole and hence cannot be generated simply by a splitting process. The most obvious model of centriole duplication is that centrioles contain an essential template structure needed to produce a new centriole, so that new centrioles can only form when nucleated by a pre-existing one, or if without a pre-existing template, only at a very much slower rate. [30; 37]

Centriole duplication is restricted not just spatially, by the influence of pre-existing centrioles, but also temporally, under the control of the cell-cycle machinery. Assembly of new centrioles begins when cells enter S phase. A ring of nine singlet microtubules (9x1), the procentriole, is formed, and subsequently extended to the nine-triplet form (9x3). These microtubules elongate and recruit further material to form the mitotic centrosome. Eventually the centriole pairs separate, forming two centrosomes. As cells enter G1 phase, mother and daughter centriole of each centrosome detach from one another, losing their perpendicular arrangement. After mitosis, the recruited pericentriolar proteins are re-sequestered into the newly formed nuclei. [37]

1.3.4 The microtubular cytoskeleton in *Toxoplasma gondii*

Different from the usual nine-triplet form in other cells, *Toxoplasma gondii* centrioles contain nine singlet microtubules with an additional central singlet microtubule. Toxoplasma centrioles do not appear to be nucleating microtubules directly, but they

may function to organize centrin fibers that in turn link the subpellicular microtubules (see section 1.3.4.1) to the apicoplast and the nucleus. [46]

Toxoplasma gondii tachyzoites have two sets of microtubules that mediate the critically important functions of polarity, shape, host cell invasion and nuclear division. They are called spindle microtubules and subpellicular microtubules. They each have their own microtubule-organizing center (MTOC). [45]

1.3.4.1   Subpellicular microtubules

The characteristic crescent shape of *Toxoplasma gondii* is maintained by an interaction between the pellicle and the underlying twenty-two subpellicular microtubules. The pellicle is composed of the plasma membrane and the closely apposed inner membrane complex (IMC) that comprises flattened vesicles. The subpellicular microtubules (app. 5μm long) have a characteristic organization and length. They are nucleated from the apical polar ring, a unique MTOC. These microtubules are critically important for shape and polarity as well as for daughter parasite budding. [36; 44; 46; 47]

1.3.4.2   Spindle microtubules

The spindle microtubules (app. 1-2μm long) function to form an intra-nuclear spindle to coordinate chromosome segregation essential for nuclear division. Spindle microtubules originate in a dense plaque structure that is embedded in the nuclear membrane adjacent to the cytoplasmic centrioles. Spindle microtubules are associated with spindle pole plaques and adjacent centrioles and are required for their proper segregation. [44; 46]

1.3.4.3   Drug interference with microtubular system

The two microtubular systems show differential drug sensitivity. This provides the key to a separate analysis of both systems and their functions. [46] When replicating parasites are treated with the microtubule disrupting drug oryzalin, nascent subpellicular microtubules are more sensitive to disruption than are spindle microtubules. At 0.5μM oryzalin daughter parasites and centrioles could still bud and divide properly, but are affected in their shape due to the loss of proper function of the subpellicular microtubule network. At 2.5μM oryzalin both systems are affected and parasites grow as large intracellular inclusions incapable of division, and centrioles duplicate continously, unchecked. Drug washout after 48 hours leads to recovery in parasites treated with 0.5μM oryzalin, indicating that chromosomes have been properly

segregated during the drug period. Parasites treated with 2μM oryzalin are unable to recover, indicating interference with correct chromosome segregation. [46] Oryzalin treatment does not inhibit Golgi growth, but duplicated Golgi cannot segregate. Structure and function of the Golgi is kept up even when the cell shape is already compromised due to loss of the integrity of subpellicular microtubules. [60; 70]

Treatment with ethalfluralin, which selectively destroys spindle microtubules, leads to the loss of Golgi, suggesting an association between Golgi and the centriole-bound spindle microtubules. [70]

1.4 Objective of the study

The aim of this study is to describe the relationship of centrosomes and the Golgi apparatus in the apicocomplexan parasite *Toxoplasma gondii* and to thereby raise implications for the nature of Golgi inheritance in the parasite and also in general.

There are currently two opposing hypotheses about the mechanism of Golgi inheritance. One claims that the Golgi is formed *de novo* from the ER. [78]. The other model claims that the Golgi is an independent organelle and that new Golgi cannot grow *de novo*, but require a template, which is presumably given by the existing Golgi apparatus. [50; 58]

Different from mammalian cells, *Toxoplasma gondii* has only a single unit Golgi. Its simplicity has been exploited previously to gain information on the process of Golgi biogenesis. [51]

Morphological EM studies have long given insight into the composition of the parasite. A characteristic spatial relation of centrioles and Golgi has been noticed, but not further studied ([69] and D.S. Roos, University of Pennsylvania, personal communication).

Interestingly, it has been shown that another organelle, the apicoplast, a plastid organelle, is closely linked to the centrosome during the duplication process. [71]

The main goal of this study is to investigate and describe the nature of the relationship between centrioles and the Golgi. As a first step, the spatial relationship of Golgi and centrosome is demonstrated with immunofluorescence techniques in fixed parasites. As a second step, these organelles are imaged in real time in living parasites. Representative Golgi and centrosome proteins are linked to fluorescent reporters. This

allows following both organelles simultaneously through the cell cycle by fluorescence time-lapse videomicroscopy. Finally, this live-cell imaging system is used to study the influence of microtubule disruptive drugs not only on the centrosomes but also on the Golgi and the relationship of both organelles to each other.

2   Materials and Methods

2.1   Materials

2.1.1   Reagents

2.1.1.1   Phosphate-buffered saline

Phosphate-buffered saline (PBS) was used as diluent. For tissue culture and preparation of cells for microscopy calcium and magnesium free PBS, "PBS-CMF", was used. For the incubation with antibodies fish skin gelatin (0.2%) was added, "PBS+FSG", to reduce non-specific background.

2.1.1.2   Minimal essential medium and Fetal bovine serum

Minimal essential medium (MEM) (cat. no. 11095-080) was obtained from Gibco/Invitrogen Corp., Carlsbad, CA, U.S.A., and supplemented with penicillin (100U/ml) and streptomycin (100μg/ml). Fetal bovine serum (FBS) (cat. no. 100-106) was obtained from Gemini Bio-Products, Woodland, CA, U.S.A. It was added to final concentrations of either 1% or 10% to MEM. Phenol red free, clear medium was used for microscopy purposes.

2.1.1.3   Ampicillin

Ampicillin was obtained from J.T.Baker, Phillipsburg, NJ, U.S.A., and diluted to 50mg/ml in dH$_2$O and ethanol (equal amounts). For use in LB medium it was diluted to a final concentration of 100μg/ml.

2.1.1.4   Triton X-100

Triton X-100, was obtained from J.T.Baker, Phillipsburg, NJ, U.S.A. Solutions (0.25% and 1%) in PBS-CMF were used to permeabilize membranes for immunostaining.

2.1.1.5   Hoechst 33342

Hoechst 33342 (bisbenzimide's ethyl ether) was obtained from Sigma-Aldrich Co., Milwaukee, WI, U.S.A., and used to stain DNA. For images of fixed parasites, cells were incubated for 5 minutes in its presence (10μg/ml). For live imaging it was added to the medium (5μg/ml) and the parasites were incubated for 10 minutes. Afterwards the

medium was exchanged and the dye was removed. Parasites were incubated for another hour and medium was exchanged again immediately prior to microscopy.

2.1.1.6   Chloramphenicol

Chloramphenicol was obtained from Sigma-Aldrich Co., Milwaukee, WI, U.S.A., and kept as a stock solution (0.105M in ethanol) at –20°C. For use in tissue culture it was diluted to the desired concentration directly in the tissue culture medium. The optimal concentration for drug selection (20µM in this case) was estimated by previous reports [54; 71] and own experience by serial dilution.

2.1.1.7   Oryzalin

The dinitroaniline herbicide oryzalin was obtained from Sigma-Aldrich Co., Milwaukee, WI, U.S.A.

2.1.1.8   Electroporation buffer

For the transformation of *Toxoplasma gondii* parasites by electroporation, a special buffer was mixed that imitated the intracellular environment. [54] The buffer contained 120mM KCl, 0.15mM $CaCl_2$, 10mM $KHPO_4/KH_2PO_4$ pH 7.6, 25mM HEPES, pH 7.6, 2mM EDTA, 5mM $MgCl_2$, 2mM ATP and 5mM glutathione (GSH) diluted in dH_2O. ATP and GSH were added immediately prior to use. Also, the buffer was re-sterilized each time it was used by filtration through a 0.22 µm pore filter.

2.1.1.9   Tris-Acetate-EDTA buffer

For 50x Tris-Acetate-EDTA buffer (TAE buffer) stock solution, 242g of tris base, 57.1ml acetic acid and 100ml 0.5M EDTA were mixed and supplemented up to a 1000ml with dH_2O. For making gels the buffer was diluted in dH_2O.

2.1.2 Host cells

Human foreskin fibroblast (HFF) cells were obtained from the American Tissue Culture Collection, Manassas, VA, U.S.A.

2.1.3 *Toxoplasma gondii* parasite lines

2.1.3.1   RH wild-type

The *Toxoplasma gondii* RH wild-type parasites were kindly provided by D.S. Roos, University of Pennsylvania, Philadelphia, PA, U.S.A.

2.1.3.2   RH centrin-EGFP

A transgenic *Toxoplasma gondii* line expressing a construct of endogenous *Toxoplasma gondii* centrin tagged to EGFP was obtained from K. Hu, University of Pennsylvania, Philadelphia, PA, U.S.A. (M. Nishi, K. Hu, J.M. Murray and D.S. Roos: "Organellar Dynamics during the Cell Cycle of *Toxoplasma gondii*", paper in progress).

2.1.3.3   RH GRASP55-YFP

The transgenic *Toxoplasma gondii* RH GRASP55-YFP parasite line and the GRASP55-YFP plasmid in a *Toxoplasma gondii* expression vector (p*tub*GRASP55-YFP/*sag*-CAT) were provided by L. Pelletier (Max Planck Institute for Molecular Cell Biology and Genetics, Dresden, Germany). This expression vector uses the *Toxoplasma gondii tub1* promoter [72] and is followed by the 340 amino-terminal amino acids of rat GRASP55 followed by the YFP coding region. [51]

2.1.3.4   RH GRASP55-mRFP/centrin-EGFP

The double stable transgenic *Toxoplasma gondii* RH GRASP55-mRFP/centrin-EGFP parasite line was made by inserting the GRASP55-mRFP plasmid into parasites from the RH centrin-EGFP line. This was done by electroporative transfection and consequent selection with chloramphenicol. (see 2.2.4 and 2.2.5)

2.1.4 Antibodies

2.1.4.1   anti-centrin antibody

The mouse monoclonal antibody against the mammalian protein centrin used in this study was obtained from J. L. Salisbury. [56] The same antibody has been characterized in *Toxoplasma gondii* previously and has been used to label their centrosomes by immunofluorescence. [60; 71]

2.1.4.2   anti-GFP antibody

The rabbit polyclonal antibody used to label GFP-tagged constructs was raised by J. Seemann (Southwestern Medical School, Dallas, Texas, U.S.A.) Since it cross-reacted with YFP- and CFP-variants, it could also be used to label YFP-tagged constructs.

2.1.4.3   Secondary antibodies

The secondary antibodies used were goat anti-mouse and goat anti-rabbit conjugated to Alexa Fluor™488 and Alexa Fluor™594 (Molecular Probes Inc., Eugene, OR, U.S.A.) They were diluted to 20µg/ml in PBS for incubation.

2.2   Methods

2.2.1   Tissue culture

2.2.1.1   Host cells

Human foreskin fibroblasts (HFF) used as host cells for the *Toxoplasma gondii* tachyzoites were grown in T-175 and T-25 flask, suspended in MEM (10% FBS) at 37°C under a 5% CO_2 atmosphere. When the cell layer reached confluence, cells were passed into new flasks following a standard procedure: the old medium was removed from the flask. The remaining cells were rinsed extensively in PBS-CMF to wash out the medium completely, because it contained a trypsine inhibitor. Hereafter the cell layer was exposed to trypsin-EDTA and incubated for 2 min at 37°C. Then the flask was removed from the incubator and gently shaken to detach all cells from the flask bottom. Fresh medium was added and the re-suspended cells were filled into new flasks at the desired dilution (usually 1ml suspension into 6 ml fresh medium).

2.2.1.2   *Toxoplasma gondii* tachyzoites

Toxoplasma gondii parasites were grown in HFF host cells in flasks containing MEM (1% FBS). When the parasites had lysed about 80% of the host cell layer and they were floating in the medium, 1ml out of the 6 ml of the medium was transferred into a flask with a fresh host cell layer, in which the medium had been exchanged from MEM (10% FBS) to MEM (1% FBS).

2.2.2 Antibody staining

To obtain images from *Toxoplasma gondii*, parasites were fixed within the host cells grown on glass coverslips by exposure to paraformaldehyde (4% in PBS-CMF) for at least 20 minutes. They were then quenched for 10 minutes in 50mM NH_4Cl made in PBS-CMF, washed three times in PBS-CMF and permeabilized by 5-minute treatment with Triton X-100 (0.25% in PBS). Hereafter they were prepared for immunostaining by washing them three times in PBS-CMF and for another five minutes in PBS+FSG.

Flipped to the reverse side onto parafilm (Parafilm M$^®$ by Pechiney Plastic Packaging, Chicago, IL, U.S.A.) with a drop of PBS+FSG containing the primary antibodies at desired dilutions, the coverslips were incubated for 20 minutes at 37°C. After rinsing them three times in PBS+FSG they were incubated with the fluorescently conjugated secondary antibodies for 20 minutes at 37°C. Optionally they were rinsed another time to be incubated with Hoechst 33342 for 5 minutes at 37°C. Before mounting the cover slips to glass slides they were rinsed a final time in PBS+FSG. They were stored in a dark, ambient place to dry.

2.2.3 Microscopy

All microscopy was performed using a Zeiss Axiovert 100M inverted microscope (Carl Zeiss, Jena, Germany). It was equipped with an Orca-100 CCD camera (1280x1022 pixels 1x1 binning, Hamamatsu Photonics Systems, Bridgewater, NJ, U.S.A.) and with a Polychrome II monochromator (TILL Photonics, Martinsried, Germany). The microscopy and imaging station was controlled with Openlab 3.0.8. (Improvision, Coventry, UK) run on a Macintosh G4 (Apple Computer, Cupertino, CA, U.S.A.). A Plan-Apochromat Ph3 100x 1.4NA objective along with selective single band-pass filters were used (Chroma Technologies, Brattleboro, VT, U.S.A.). For visualization of EGFP and mRFP in the same microscopy field a custom filter block was used (AHF Analysetechnik, Tübingen, Germany).

For live cell imaging parasites were grown in HFF cells on glass bottom dishes (#1.5, MatTek Corporation, Ashland, MA, U.S.A.). They were put into a CO_2 diffusion chamber and an incubator was placed around the microscope to provide an ambient atmosphere. During the imaging HEPES buffer was added to the medium.

2.2.4 *Toxoplasma gondii* transfection

For transfection of *Toxoplasma gondii* parasites about 50 µg of the desired plasmid DNA had to be precipitated. To this end the DNA was mixed with 3M sodium acetate (10% of the volume of the DNA solution, pH 5.0) and vortexed. It was then washed with pure ethanol (2.5fold volume of the DNA solution, -20°C) and vortexed again. Hereafter it was centrifuged (Eppendorf 5415 D centrifuge, Eppendorf, Hamburg, Germany) at 13,200 rpm for 15 minutes, then washed twice with 1ml ethanol (70% in distilled water) and centrifuged again. The ethanol supernatant was removed completely and the DNA pellet air-dried just shortly. The DNA was then resuspended in 100µl electroporation buffer.

Meanwhile the parasites (app. 5×10^7) were harvested from the flasks and filtered through 3µm filters (Nuclepore Track-Etch Membrane, Whatman Inc., Sanford, ME, U.S.A.) to remove all cell debris. They were spun down at 1250g and 37°C for 5 minutes (centrifuge 5810 R, Eppendorf, Hamburg, Germany), supernatant was removed and the parasites re-suspended in 300µl electroporation buffer.

For electroporation, resuspended DNA and parasites (adding up to 400µl volume) were filled into a 0.2cm gap electroporation cuvette (cat. no. 165-2086, BioRad, Hercules, CA, U.S.A.) and gently mixed by pipetting up and down. They were put into an electroporation chamber connected to the Gene Pulser II device (BioRad, Hercules, CA, U.S.A.), which was set to 1.5kV, 25µF and time constant 0.2msec, as previously described. [54] They were pulsed once and then kept in the chamber for 10 minutes to recover. After that, the parasites were inoculated onto fresh host cell monolayers and cultivated in fresh MEM (1% FBS).

2.2.5 Drug pressure selection

For the generation of a transgenic double-stable parasite line, it was necessary to grow transformed parasites in a selective environment in which only those, that took up the plasmid DNA and incorporated it into their genome, could survive. Since the centrin-EGFP construct was already stably expressed in the specific parasite line, only the incorporation of the second construct, the GRASP55-mRFP (see section 3.3), had to be ensured. The *Toxoplasma gondii* expression vector that the GRASP55-mRFP construct had been cloned into also contained the gene for the chloramphenicol acetyl transferase

(CAT). CAT provides resistance against the drug chloramphenicol. Hence chloramphenicol was added to the medium after the transformation. The optimal concentration of drug, meaning that neither all parasites were killed nor those without the plasmid could survive, was found to be 20 µM. The concentration had been estimated by other reports and then been checked by serial dilution. [54; 71]

Under the drug treatment the parasite replication slowed down after the first round of replication. Whereas usually the parasites lysed the host cell layer of their T25 flasks within two days, it took them a week for the second round and nearly two weeks for the third. After those three rounds all parasites were still positive for the GRASP55-mRFP marker, as checked by fluorescence microscopy. The drug treatment was continued for another month, while the replication time of the parasites went back to normal values.

After the additional month the drug was removed from the medium. Parasites were regularly checked by fluorescence microscopy and a sample population was frozen down as a safety backup.

2.2.6 Plasmid DNA preparation

For the amplification of plasmid DNA QIAfilter™ Plasmid Maxi Kits and QIAprep® Spin Miniprep Kits (Cat. No. 12262 and 27106, QIAGEN, Valencia, CA, U.S.A.) were used according to the provided handbooks, modified slightly to the necessities of the laboratory equipment. The Luria-Bertani (LB) medium was mixed in a shared facility of the university. Certomat BSI (B. Braun Biotech Inc., Allentown, PA, U.S.A.) was used as a shaking incubator in which the flasks could be shaken continuously at 250 rpm and at 37°C. Centrifuges used for the various steps were Allegra™ 6R Centrifuge, Avanti Centrifuge J-20 I (both Beckman Coulter, Fullerton, CA, U.S.A.), Biofuge fresco (Heraeus, Hanau, Germany) and Eppendorf Centrifuge 5415D (Eppendorf, Hamburg, Germany).

2.2.7 Gel electrophoresis

To separate or measure DNA fragments, agarose gel electrophoresis was performed. Gels were made by melting agarose in TAE buffer (app. 1g agarose per 100ml of buffer). Ethidium bromide was added to the gel at a concentration of 1µg/ml. The gel was cooled down a little and poured into a plastic cassette with a comb to cool down

completely and polymerize. Upon polymerization, the comb was removed and the gel was immersed in TAE buffer. Approximately 20-30µl of the DNA sample plus 0.1vol loading buffer were loaded into each well. At least one lane was filled with a DNA ladder, indicating the fragment size (1kb DNA ladder, cat. no. 15615-016, Invitrogen, Carlsbad, CA, U.S.A.). The gel was then run at 120V for 30 to 60 minutes, depending on the individual needs. Gels were evaluated in a dark room on a UV light transilluminator and photographed with a Polaroid MP-3 Land Camera.

2.2.8 Restriction endonucleases

When desired, DNA was cleaved by restriction endonucleases to get the required fragments or overhangs. To this end 1µg of DNA (calculated from optical density of the solution) was digested in the presence of 5 Units of the specific enzymes per µg DNA. Sample volume was adjusted with dH_2O and usually incubated for one hour at the temperature recommended by the manufacturer. All restriction endonucleases and corresponding buffers were purchased from New England Biolabs, Inc., Beverly, MA, U.S.A.

2.2.9 Polymerase Chain Reaction (PCR)

PCRs were performed in a MiniCycler™ (MJ research, Inc., Watertown, MA, U.S.A.) or GeneAmp® PCR system 9700 (Applied Biosystems, Foster City, CA, U.S.A.) Following the standard protocol all components were pre-mixed in PCR tubes: template, 5µl 10x buffer (matching the polymerase used), 2µl of a dNTP mix (containing 10µM of each nucleotide), the forward and reverse primer (at a 10µM concentration of each), dH_2O (up to a final volume of 50µl) and the DNA-polymerase (either Taq or PfU). The tubes were overlaid with an oil drop or sealed and put into the thermocycler. The standard program was: step 1) denature for 2 minutes at 94°C, step 2) denature for 30 seconds at 95°C, step 3) annealing for thirty seconds at 70°C, step 4) extension for 1 minute at 72°C, step 5) go back to step 2) for thirty more times, step 6) extra extension for 10 minutes at 72°C and step 7) storage at 4°C until the end. The temperatures and times of the program were specifically modified for each PCR. They were adjusted to the polymerase, the expected size of the DNA fragment, the

characteristics of the primers and on the intention of the PCR, such as screening or highly precise and specific amplification of a fragment.

2.2.10 DNA sequencing

Sequencing of DNA samples was performed by the W.M. Keck Foundation Biotechnology Resource Laboratory, Yale University, New Haven, CT, U.S.A. Samples were submitted following a standard protocol: 600ng plasmid DNA, with 2µl of 4µM primer or 0.8µl of 10 µM primer and dH$_2$O up to a final volume of 24µl. Sequencing results were obtained through the Yale University data network and processed with DNAStar® or similar software.

Table 1 – Sequencing primers. These primers were used to sequence the obtained p*tub*GRASP55-mRFP/(YFP)/*sag*-CAT construct.

"VIF"	5'-CACAATCACCTTGTGTGAAGTTCTTGCG-3'
"VIR"	5'-ACGTCGCCGTCCAGCTCGAC-3'
"RSF"	5'-ATGGCCTCCTCCGAGGACGTCATCAAGG-3'
"RSF2"	5'-GAGCGGATGTACCCCGAGGACGGCG-3'
"RSR1"	5'-TTAGGCGCCGGTGGAGTGGCGGCCC-3'
"RSR2"	5'-CACCTTCAGCTTGGCGGTCTGGGTGCC-3'

To confirm the integrity of the p*tub*GRASP55-mRFP/(YFP)/*sag*-CAT construct, it was sequenced. (for details see section 2.1.3.4) For the sequencing several forward and reverse primers were used (see Table 1) and all results were combined using DNAStar® software to give one reliable result. The sequence obtained was compared to the mRFP sequence from the online database using the online BLAST program provided by the NCBI of the NLM, Washington D.C., U.S.A. [2] The sample showed a perfect match (see Figure 2), and was used for all further experiments.

```
  >gi|214464837|gb|AF506027.1|    Synthetic construct monomeric red fluorescent protein gene,
           complete cds
           Length = 678

 Score = 1344 bits (678), Expect = 0.0
 Identities = 678/678 (100%)
 Strand = Plus / Plus


Query: 1     atggcctcctccgaggacgtcatcaaggagttcatgcgcttcaaggtgcgcatggagggc 60
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 1     atggcctcctccgaggacgtcatcaaggagttcatgcgcttcaaggtgcgcatggagggc 60


Query: 61    tccgtgaacggccacgagttcgagatcgagggcgagggcgagggccgcccctacgagggc 120
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 61    tccgtgaacggccacgagttcgagatcgagggcgagggcgagggccgcccctacgagggc 120


Query: 121   acccagaccgccaagctgaaggtgaccaagggcggccccctgcccttcgcctgggacatc 180
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 121   acccagaccgccaagctgaaggtgaccaagggcggccccctgcccttcgcctgggacatc 180


Query: 181   ctgtcccctcagttccagtacggctccaaggcctacgtgaagcaccccgccgacatcccc 240
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 181   ctgtcccctcagttccagtacggctccaaggcctacgtgaagcaccccgccgacatcccc 240


Query: 241   gactacttgaagctgtccttccccgagggcttcaagtgggagcgcgtgatgaacttcgag 300
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 241   gactacttgaagctgtccttccccgagggcttcaagtgggagcgcgtgatgaacttcgag 300


Query: 301   gacggcggcgtggtgaccgtgacccaggactcctccctgcaggacggcgagttcatctac 360
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 301   gacggcggcgtggtgaccgtgacccaggactcctccctgcaggacggcgagttcatctac 360


Query: 361   aaggtgaagctgcgcggcaccaacttcccctccgacggccccgtaatgcagaagaagacc 420
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 361   aaggtgaagctgcgcggcaccaacttcccctccgacggccccgtaatgcagaagaagacc 420


Query: 421   atgggctgggaggcctccaccgagcggatgtaccccgaggacggcgccctgaagggcgag 480
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 421   atgggctgggaggcctccaccgagcggatgtaccccgaggacggcgccctgaagggcgag 480


Query: 481   atcaagatggaggctgaagctgaaggacggcggccactacgacgccgaggtcaagaccacc 540
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 481   atcaagatggaggctgaagctgaaggacggcggccactacgacgccgaggtcaagaccacc 540


Query: 541   tacatggccaagaagcccgtgcagctgcccggcgcctacaagaccgacatcaagctggac 600
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 541   tacatggccaagaagcccgtgcagctgcccggcgcctacaagaccgacatcaagctggac 600


Query: 601   atcacctcccacaacgaggactacaccatcgtggaacagtacgagcgcgccgagggccgc 660
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 601   atcacctcccacaacgaggactacaccatcgtggaacagtacgagcgcgccgagggccgc 660


Query: 661   cactccaccggcgcctaa 678
             ||||||||||||||||||
Sbjct: 661   cactccaccggcgcctaa 678
```

Figure 2 – GRASP55-mRFP construct sequence matches database sequence. The sequence of the mRFP part of GRASP55-mRFP was obtained by combining the sequencing results with different primers. The computed sequence was run through the NCBI online BLAST program and compared to the database sequence of mRFP, showing a perfect match.

3   Results

3.1   Observations by immunofluorescence microscopy

Earlier studies had pointed to a spatial relation between the Golgi apparatus and the centrosomes in *Toxoplasma gondii*. To follow up these observations triple labeling studies were performed to simultaneously visualize the Golgi, the centrosomes and the nucleus, as a fixed landmark in the parasite. To this end, a parasite line (see section 2.1.3.3) stably expressing GRASP55-YFP, a marker of medial Golgi cisternae, was used. Polyclonal rabbit anti-GFP antibodies (see section 2.1.4.2) were used to label the GRASP55-YFP construct, indicating the Golgi apparatus. Monoclonal mouse anti-centrin antibodies (see section 2.1.4) were used to label the centrosomes. Alexa Fluor™ 594 goat anti-mouse and Alexa Fluor™ 488 goat anti-rabbit were used as secondary antibodies and Hoechst 33342 was used to label the nuclei.

The initial images revealed a close relationship between both organelles (see Figure 3). After optimizing the conditions of immunofluorescence parasites from an unsynchronized population were studied to quantify the findings. Vacuoles were chosen randomly, parasites were counted and categorized by number of Golgi and centrosomes and also divided into two groups depending on their spatial relation to each other. Parasites that showed centrosomes adjacent to or co-localizing with the Golgi were put into the first group referred to as "together". Those in which such close spatial relationship was not found, were put into the second category referred to as "apart".
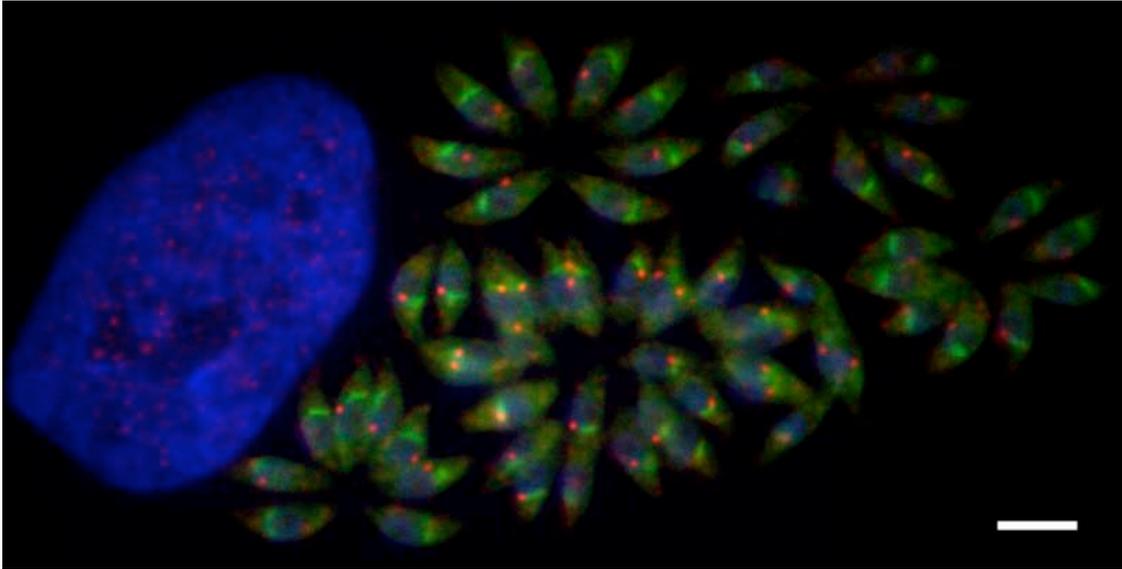
Figure 3 – Immunofluorescence microscopy image of several *Toxoplasma gondii* vacuoles in an HFF host cell. Nuclei of both *Toxoplasma gondii* parasites as well as the host cell (big one, left side) are stained with Hoechst 33342 (blue). Centrosomes (red) are labeled with secondary antibodies to anti-centrin antibodies, Golgi (green) with secondary antibodies to anti-GFP antibodies recognizing the overexpressed GRASP55-YFP. Some parasites show a spatial relationship between the Golgi and the centrosome. Other parasites display Golgi and centrosomes well separated. Scale bar is 5 μm.

The total number of parasites counted was 554. 452 out of these (81.59%) fit the category "together", 102 (18.41%) fit the category "apart".

Out of the parasites of the first category, 294 showed one Golgi and one centrosome, 123 showed two Golgi and two centrosomes, 33 showed two centrosomes, but only one Golgi and 2 showed two Golgi but only one centrosome.

Out of the second category, 55 showed one Golgi and one centrosome, 39 showed one Golgi but two centrosomes, 2 showed two Golgi and one centrosome and 6 showed two Golgi and two centrosomes.

Table 2 - Golgi to centrosome relation. In a random, asynchronous parasite population parasites were screened and put into categories depending on the number of Golgi and centrosomes and the spatial relation to each other.

no. of centrosomes	**no. of Golgi**			
	together	**1**	**2**	
	1	294	2	
	2	33	123	$n_t = 452$
	apart	1	2	
	1	55	2	
	2	39	6	$n_a = 102$
				$n_{total} = 554$

At the moment of fixation in 81.59% of the parasites there was a physical relation between the Golgi and the centrosome. Since the parasite population was unsynchronized and therefore random in regard to their stage in the life cycle, this should correlate to the percentage of time in the parasite's cell cycle.

Golgi and centrosomes seemed to duplicate around the same time, since the number of their copies in the parasites correlated rather well. Centrosomes seemed to duplicate a little earlier, however, since there were more cases with duplicated centrosomes but still only one Golgi, especially in the "apart" group.

Since these data relied on still images of fixed cells, no further conclusions could be drawn. It could not be determined whether the other 18.41% would never show such a relation or if they just did not show it at the moment of fixation. It could only be speculated on the order of events.

To get information concerning these questions it was necessary to develop a system in which it would be possible to study living parasites and to follow their Golgi and centrosomes through the cell cycle.

3.2 Centrin-EGFP is a suitable centrosome marker

A parasite line expressing endogenous *Toxoplasma gondii* centrin tagged to EGFP was developed by a collaborating laboratory (RH centrin-EGFP, section 2.1.3.2). To confirm that the centrin signal would be useful to visualize the centrosomes in living parasites, it was compared to the signal derived from stainings with the well-characterized anti-centrin antibody in fixed parasites.

In a co-localization study the parasites were co-stained using the monoclonal mouse antibody against centrin and the polyclonal rabbit antibody against GFP, to increase the centrin-EGFP signal. Alexa Fluor™ 594 goat anti-mouse and Alexa Fluor™ 488 goat anti-rabbit were used as secondary antibodies.
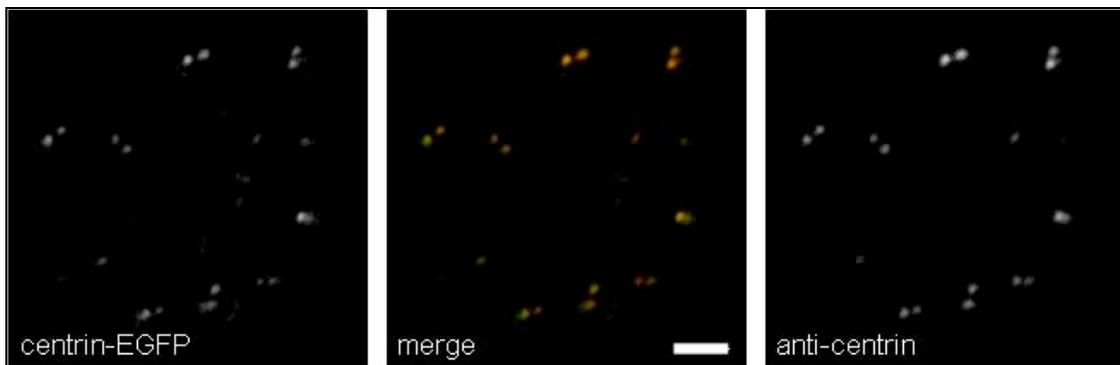
Figure 4 - Colocalization of centrin-EGFP and anti-centrin signals. Representative immunofluorescence image of parasites showing that the centrin-EGFP signal (left and green) colocalizes perfectly with the established antibody patterns (right and red). Scale bar is 5 μm.

As depicted in Figure 4, the signal obtained from the anti-centrin antibody marking both the endogenous and the EGFP-tagged version of centrin colocalized with the signal from the anti-GFP antibody. There were no extra signals in the anti-centrin channel. This proved that there was no extra occurrence of endogenous centrin in spots other than those labeled with the overexpressed centrin-EGFP anyway. Hence it could be concluded that cloned and overexpressed endogenous *Toxoplasma gondii* centrin tagged to EGFP was equally suitable to be used as a centrosome marker. Moreover it had the benefit of allowing live imaging.

3.3   Golgi marker GRASP55-mRFP

3.3.1 Generation of a GRASP55-mRFP construct

To observe the centrosome and the Golgi simultaneously in living parasites, both organelles had to be linked to fluorescent reporters. To accomplish this, centrin-EGFP in the RH centrin-EGFP line was constructed. For the Golgi the existing plasmid, GRASP55-YFP, had to be modified, because the excitation/emission wavelengths of EGFP and YFP would have been to close to discriminate by fluorescence microscopy. A suitable fluorescent reporter protein was found in the synthetic monomeric red fluorescent protein, mRFP (GenBank database accession no. **AF506027**). [7] There were no data on its use in *Toxoplasma gondii*.
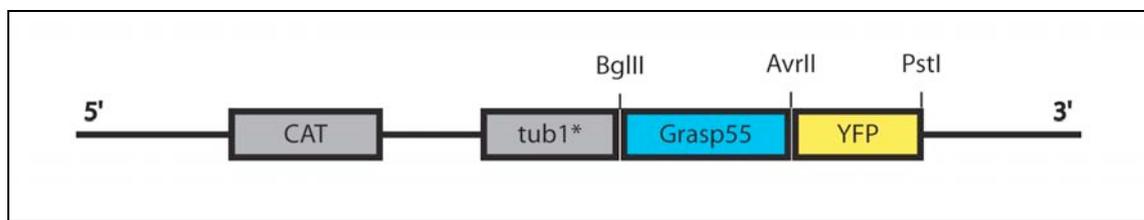
Figure 5 – p*tub*GRASP55-YFP/*sag*-CAT expression vector. This expression vector is driven by the tub1 promoter. Downstream are the GRASP55 and the YFP coding regions, seperated by an *AvrII* endonuclease restriction site. Note that the vector also contains a gene for chloramphenicol transferase (CAT), providing resistance against chloramphenicol. This vector had to be modified because the YFP excitation/emission wavelength would have been to close to that of EGFP used to label centrin and thereby the centrosomes.

p*tub*GRASP55-YFP/*sag*-CAT (Figure 5) was designed as an easily adjustable *Toxoplasma gondii* expression vector. [51] It had unique restriction endonuclease cleavage sites between all variable components (promoter, protein of interest, fluorescent reporter) and the rest of the vector. The GRASP55 sequence contained an additional *PstI* site, making it impossible to cut out and exchange the YFP region for mRFP by double digestion (*AvrII* and *PstI*) and religation. This would have resulted in another cleavage within the GRASP55 coding region.
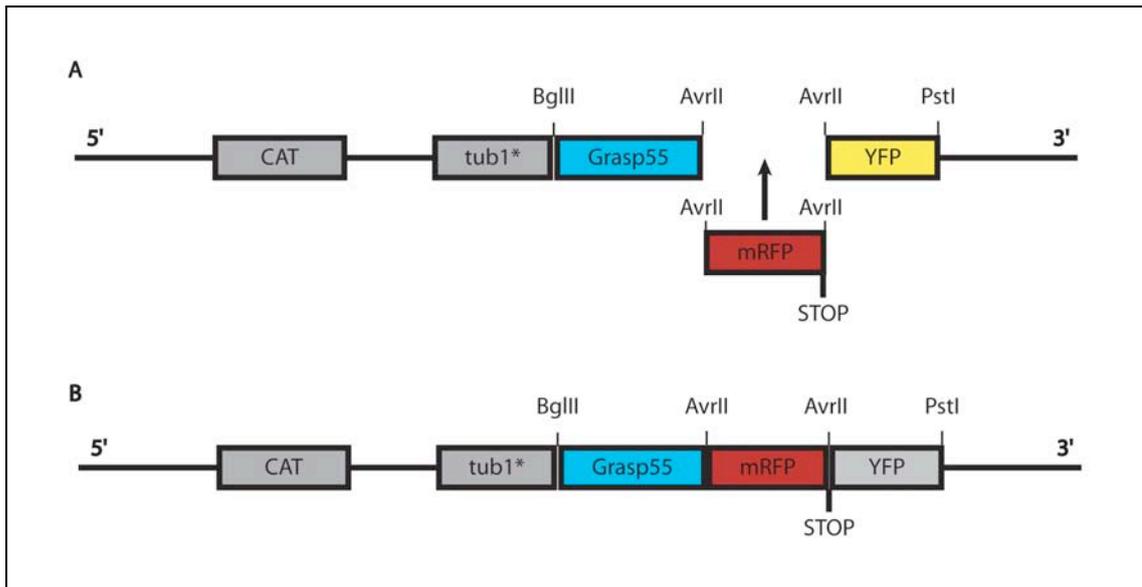
Figure 6 – Construction of the p*tub*GRASP55-mRFP/(YFP)/*sag*-CAT expression vector. The p*tub*GRASP55-YFP/*sag*-CAT expression vector was opened up by *AvrII* digestion and the mRFP DNA with *AvrII* overhangs at both ends was inserted by religation. Note the additional STOP codon that prevents the expression of YFP.

The cDNA of mRFP was provided embedded in the pRSETB vector (Invitrogen, Carlsbad, CA, U.S.A.) by R.Y. Tsien (University of California, San Diego, CA, U.S.A.). It was amplified by PCR (see section 2.2.9) from the provided plasmid. To clone the mRFP between the GRASP55 and YFP DNA, it was necessary to introduce *AvrII* endonuclease restriction sites (CCTAGG) to both ends of the mRFP cDNA. This was done by using specific primers for PCR (forward primer, "RFPforward": 5'-ATGACGATCCTAGGACA**atg**gcctcctccgagg-3'; reverse primer, "RFPreverse": 5'-GGATCAAGCCCTAGGTTA**tta**ggcgccggtggagtgg-3'). Moreover, the reverse primer provided the cDNA with an additional stop codon directly downstream of the mRFP with its own stop codon, ensuring that the YFP would not be expressed. (see Figure 6)
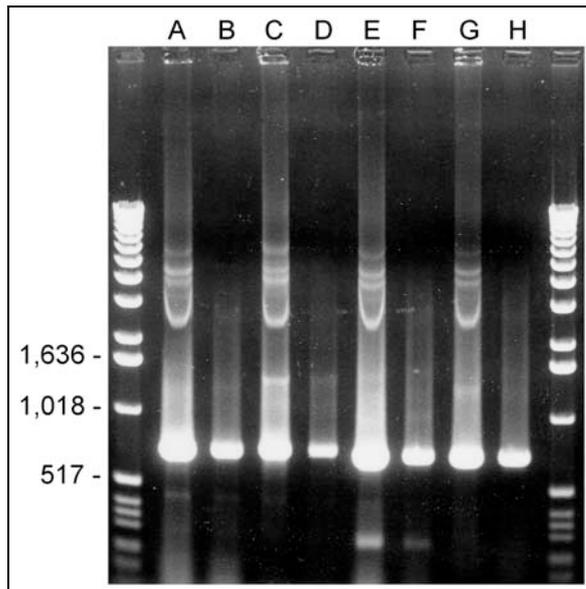
Figure 7 – mRFP ampflification by PCR. This photograph shows the results of a gel run with different samples of mRFP cDNA (size: 678bp) that had been PCR amplified with the necessary overhangs from a small probe of mRFP embedded in a commercial vector.

The resulting PCR product (Figure 7, lane "B", band of appr. 678bp) was gel-extracted, ligated into the pGEM®-T Easy Vector (Promega Corp., Madison, WI, U.S.A.) and the ligation product was then transformed into XL1-Blue Supercompetent Cells according to the provided manual (Stratagene, La Jolla, CA, U.S.A.). Cells were plated on selective LB agar. Overnight incubation yielded numerous colonies, which were checked by PCR for positive colonies that had taken up the mRFP insert. (see Figure 8)
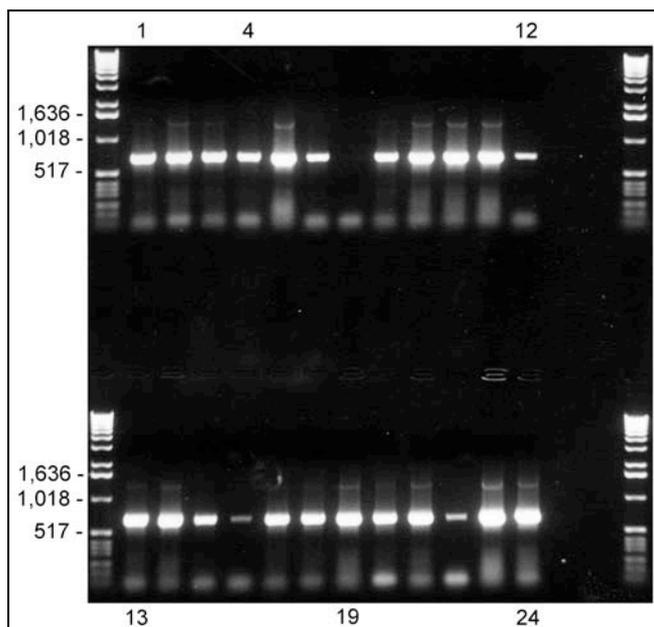
Figure 8 – PCR screening of overnight colonies. Overnight incubation of Supercompetent cells containing the mRFP ligation yielded numerous colonies. To find a clone that had taken up the plasmid correctly, small amounts from the colonies were used to perform a PCR screen using the same primers that had been used to amplify the construct from the sample probe. Except for lane 7 all show a bright band of the expected size.

One of the positive colonies (Figure 8, lane "19") was used to do a miniprep (see section 2.2.6) to amplify the plasmid DNA. The yield of this miniprep was then digested with *AvrII* restriction endonuclease to specifically open up the plasmid and to cut out the mRFP cDNA with the desired *AvrII* overhangs at both ends. (see section 2.2.8) After digestion the samples were run on a gel (see section 2.2.7) and the mRFP band was extracted from the gel (QIAquick® Gel Extraction Kit, Cat. No. 28704, QIAGEN, Valencia, CA, U.S.A.) yielding the desired mRFP copy (Figure 9 and Figure 10, lane "19").
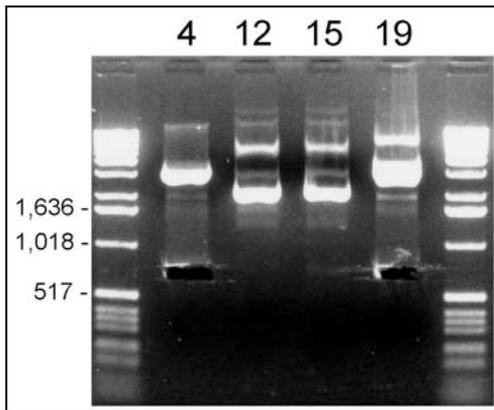
Figure 9 – Gel with digested minipreps samples. The miniprep product of the positive clone "19" was run on a gel. It was then extracted from it and purified, yielding mRFP cDNA with the desired *AvrII* overhangs.

By overnight ligation the mRFP cDNA fragment was then introduced into the p*tub*GRASP55-YFP/*sag*-CAT vector plasmid, which had been amplified previously and opened up by *AvrII* digestion at the corresponding enzyme restriction site in between the GRASP55 and the YFP coding region (Figure 10, band in lane "V").
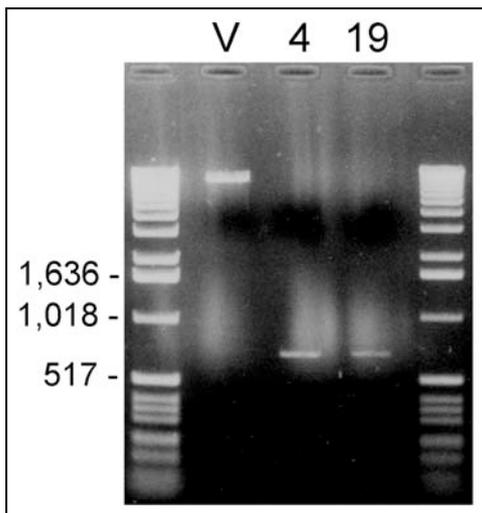
Figure 10 – Prepared vector plasmid and mRFP insert. This gel was run with small amounts of the p*tub*GRASP55-YFP/*sag*-CAT vector, that had been opened up and linearized by *AvrII* digestion, "V", and with a sample of the mRFP fragment, "19".

The ligation was checked by PCR screening (Figure 11). For this, primers were used that could specifically detect the correct incorporation of the mRFP cDNA: the forward

primer ("RFPforward") was within the mRFP region, the reverse primer ("VIR ": 5'-ACGTCGCCGTCCAGCTCGAC-3') was inside the down-stream YFP region of the p*tub*GRASP55-YFP/*sag*-CAT expression vector. By this not only incorporation of the mRFP was guaranteed, but also its proper orientation. (Due to the symmetric *AvrII* ends of the mRFP fragment it could have been inserted inversely.)
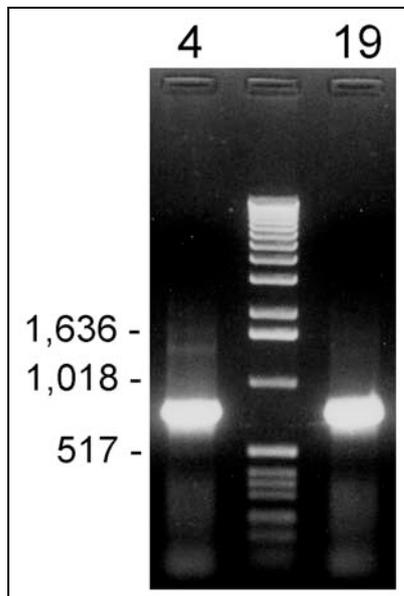

Figure 11 – PCR screen of the vector/insert ligations. The ligation of the mRFP fragment with the p*tub*GRASP55-YFP/*sag*-CAT vector was checked by PCR screening with a specific pair of primers. The PCR products were run on a gel. The results are shown. A bright band of appr. 650bp correlates to the estimated distance between the two primer sites within the vector.

The ligations of the mRFP fragment with the vector showed a band of the expected size (see Figure 11) and sample "19" was used to be transformed into XL1-Blue Supercompetent Cells. It was plated and incubated overnight. The colonies were PCR screened with the same pair of primers ("RFPforward" and "VIR") and one positive colony ("19B") was used to do a maxiprep (see section 2.2.6) to yield large amounts of the plasmid (see Figure 12).
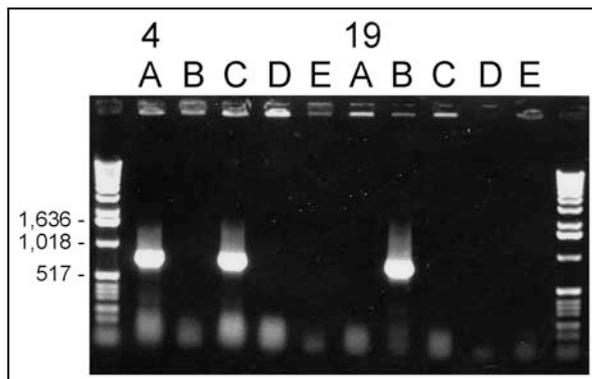
Figure 12 – PCR screen of XL-1 Blue colonies for positive clones containing the desired construct.
Only one colony from the cells transformed with mRFP sample 19 was positive (band in lane 19B).

For confirmation, the large prep product was sequenced using specific forward and reverse primers within the mRFP region or in the adjacent parts of the expression vector. (Table 1, page 22) The results were put together with DNAStar® software, and blasted through BLAST (NCBI, NLM, Washington D.C., U.S.A.) [2].

3.3.2 GRASP55-mRFP proves to be a suitable Golgi marker

RH wildtype parasites were transiently transfected with the new construct. Parasites were checked by fluorescence microscopy 24h post transfection with p*tub*GRASP55-mRFP/(YFP)/*sag*-CAT. The parasites expressed GRASP55-mRFP, displaying their Golgi marked in red. As expected, no YFP signal was detected, neither by direct nor by anti-GFP antibody mediated fluorescence microscopy.

To assure that the new construct would properly label the Golgi apparatus it was used to transiently transfect the RH GRASP55-YFP line (see section 2.1.3.3). The mRFP fluorescence colocalized precisely with the YFP signal at all times during the cell cycle (see Figure 13), validating its use as a Golgi marker. Moreover it could be observed that in parasites that expressed the GRASP55-mRFP the signal of the original GRASP55-YFP became weaker, suggesting that both proteins were competing for the same localization.
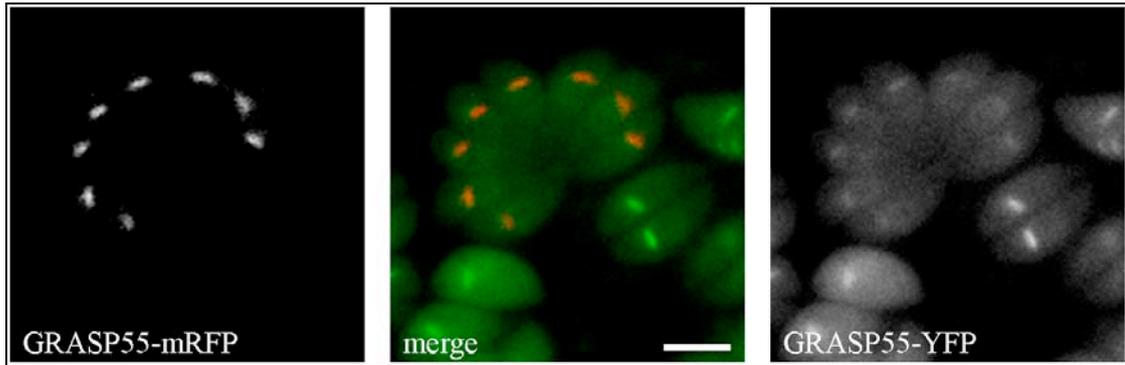
Figure 13 – Colocalization of GRASP55-mRFP and GRASP55-YFP. Representative immunofluorescence microscopy image showing the colocalization (merge in the middle) of GRASP55-mRFP (left and red) with GRASP55-YFP (right and green). Note that only one vacuole expresses GRASP55-mRFP. Scale bar is 5µm.

3.4 Transient plasmid transfection into RH centrin-EGFP parasite line

After constructing GRASP55-mRFP and showing its utility as a Golgi marker in living parasites, it was tested in the RH centrin-EGFP parasite line. It was unclear whether the parasites would survive a second overexpressed protein, linked to yet another fluorescent reporter.

The transient transfection, following the standard protocol, led to a transfection rate of approximately five percent. Many parasites died very rapidly and those surviving one or two generations did not display the Golgi marker anymore. This could be for two reasons. Either the plasmid was not permanently taken up into the parasite genome or those parasites that had taken up the construct were not viable afterwards.

However, following transfection, both centrosomes and the Golgi could be observed in those parasites simultaneously for a short period, marked by two fluorescent proteins whose excitation / emission wavelengths were far enough apart from one another to discriminate.

The observations in this live cell system were similar to the results in the fixed and antibody stained parasites. Moreover the ability to follow living parasites through part of the life cycle led to new insights into the dynamics of the relationship of the centrosome to the Golgi. This was only limited by the sensitivity of the parasites to the

high energy light, that they were exposed to during imaging, and the suboptimal and life-limiting conditions under the microscope.

The observations in these transiently transfected parasites suggested that the two organelles followed reproducable patterns throughout the life cycle. During and right after cytokinesis a single centrosome was closely attached to a single-copy Golgi. The centrosome then detached and moved to the basal part. It duplicated there and moved back up to the Golgi, which had started to duplicate as well. For daughter cell formation and cytokinesis, the duplicated Golgi and centrosomes lined up in a very distinct manner. (Figure 14)
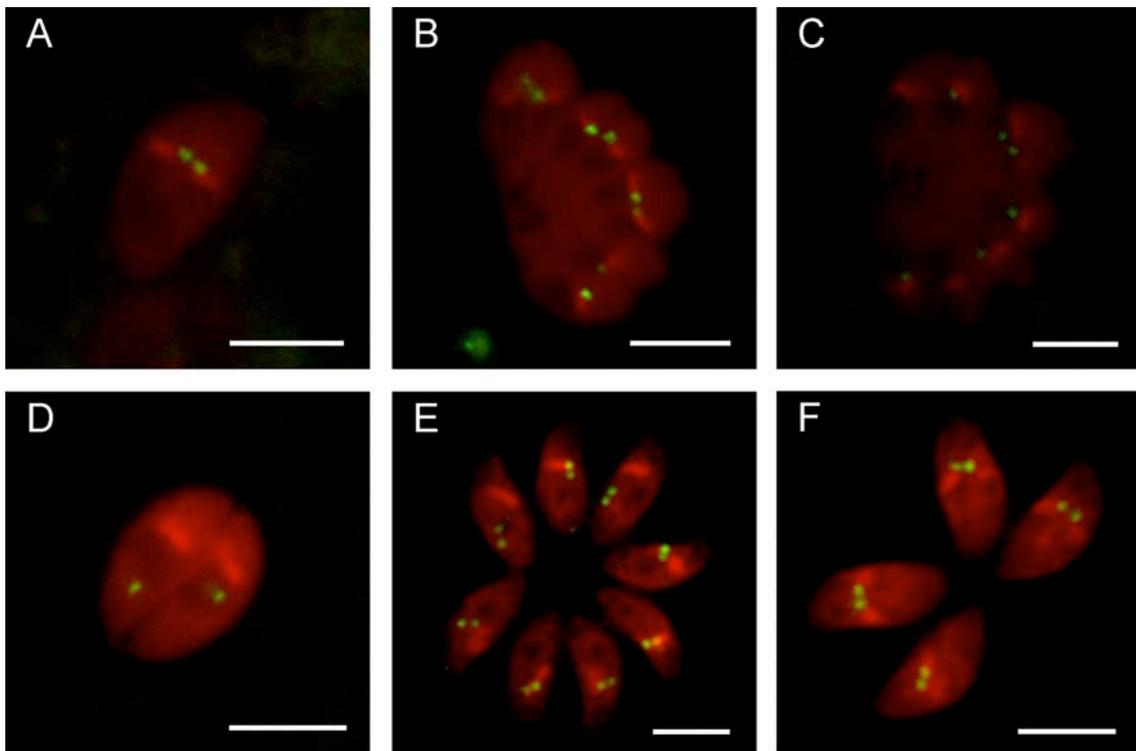

Figure 14 - Representative fluorescence microscopy images of parasites in different stages of the replication cycle. Images were obtained by fluorescence microscopy of transiently transfected parasites. The Golgi are marked in red by GRASP55mRFP, the centrosomes are marked in green with centrin-EGFP. During cytokinesis (A) and right afterwards (B) a single centrosome was closely attached to the single-copy Golgi. Later the centrosome appears to be detached from the Golgi (C). In a later stage the centrosome is observed at the basal part of the parasite (D). There it duplicate before it moves back to the Golgi, which had also begun to duplicate (E). For parasite division the duplicated Golgi and centrosomes line up with the two centrosomes in between the two Golgi (F). Scale bars are 5µm.

3.5 RH GRASP55-mRFP/centrin-EGFP double stable parasite line

3.5.1 Generation of a double stable parasite line

Our results with the transiently transfected parasites prompted us to produce a parasite line stably expressing both centrin-EGFP and GRASP55-mRFP. To this end, parasites from the stable centrin-EGFP line were transfected under standard conditions with the GRASP55-mRFP plasmid. Since this vector includes a gene that provides resistance against chloramphenicol, parasites were exposed to 20µM chloramphenicol in their medium. Therefore, only those parasites that internalized the vector with the resistance gene and integrated it into their DNA were viable. Drug treatment continued for nearly two months to strictly select for positive survivors. At each passage, a small number of parasites were used to check for the permanence of the fluorescent signal. After seven weeks the drug was removed and parasites were grown in regular medium. Even after a number of passages, parasites were positive for both markers, indicating that they had permanently taken up the construct.

3.5.2 Observations in the double stable line

The generation of this double stable line offered further insight into the order and dynamics of the Golgi / centrosome relation. Although a full life cycle could not be imaged, because of technical limitations (see section 3.4), it was still possible to follow the parasites for some period and to study the order of events, which was impossible to achieve in fixed images of the parasites. Moreover, it was possible to look at the transitions from one stage to another. By this, information could be combined to shed light on the order of events throughout the whole life cycle.

During most of the replication cycle, the centrosomes were in close proximity to the Golgi apparatus attaching to one end of the stack. Immediately after mitosis and formation of the two daughter parasites, the centrosome was located close to one end of the Golgi apparatus. (stage 1, see Figure 15) This close association was kept up for nearly half of the parasite life cycle. The centrosome then detached from the Golgi apparatus and moved down towards the basal side of the parasite (stage 2, see Figure 15), where it duplicated (stage 3, see Figure 15). After duplication, the two centrosomes

moved back to the Golgi apparatus, which also had duplicated and begun to separate. (stage 4, see Figure 15) At this stage the two Golgi and the centrosomes were very close to each other. For most of mitosis and cytokinesis, the two centrosomes were found attached to the inner sides of the separating Golgi, as if pushing the two Golgi apart. (stage 5, see Figure 15)
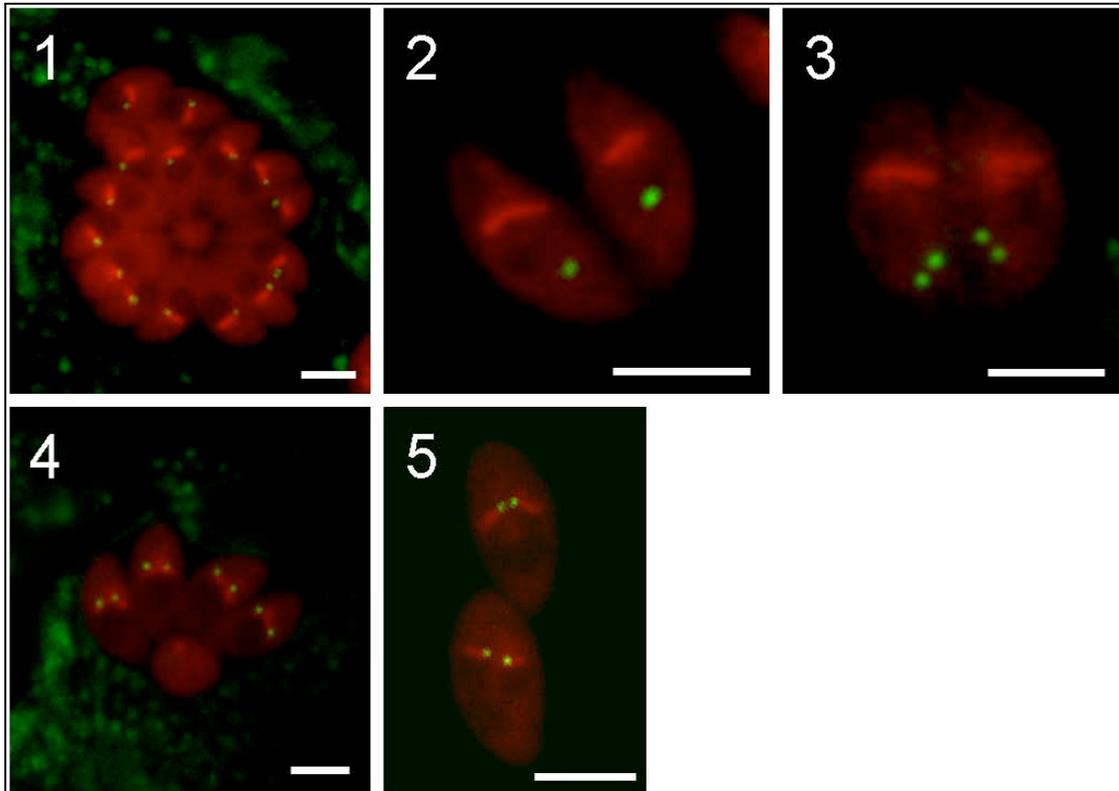

Figure 15 – Stages in the Toxoplasma gondii cell cycle. Representative fluorescence microscopy images of parasites in the different stages of the cell cycle. In stage 1 the centrosomes (green) were closely attached to one side of the Golgi (red). At stage 2 the centrosomes moved to the basal part of the parasite, where they duplicated, stage 3. The duplicated centrosomes move back up to the Golgi which showed signs of medial fission. For cytokinesis, stage 5, centrosomes and Golgi lined up in a very distinct manner. The upcoming cell division can be assumed from the U-shaped nuclei (shadow in the mRFP background). Note the non-specific background. It was due to autofluoresence of the HFF host cells. Also note the residual bodies displayed at the center of the rosettes in images 1 and 4. All scale bars are 5μm.

Since full-length life cycle observations were not possible, parasites from an unsynchronized population were randomly screened. They were counted and put into categories depending on the stage they were in. Thereby the relative length of each of the stages could be estimated. (Table 3)

Table 3 – Distribution of parasites to the different stages in the cell cycle.

stage	n	%
1	252	43.75
2	81	14.06
3	56	9.72
4	85	14.76
5	102	17.71
	n_{total}=576	100
	$n_{together}$=439	76.22%
	n_{apart}=137	23.78%

Out of a total number of 576 parasites counted, 252 (43.75%) were in stage 1, 81 (14.06%) were in stage 2, 56 (9.72%) parasites were in stage 3, 85 (14.76%) were in stage 4 and 102 (17.71) were in stage 5. A spatial relation between the Golgi and the centrosomes was found in stages 1, 4 and 5. This amounts to 439 (76.22%) parasites. 137 (23.78%) parasites did not show the centrosomes close to the Golgi but rather away from it in the basal part of the organism.

3.5.3 Drug interference

To check the effect of interference with the microtubular system on the centrosomes, the Golgi and their relationship to each other, parasites were treated with the dinitroaniline herbicide oryzalin (1µM). This drug acts by disrupting microtubules. It arrested cells mostly at a stage where large Golgi stacks were found closely associated with a pair of non-segregated centrosomes in the middle (see Figure 16).
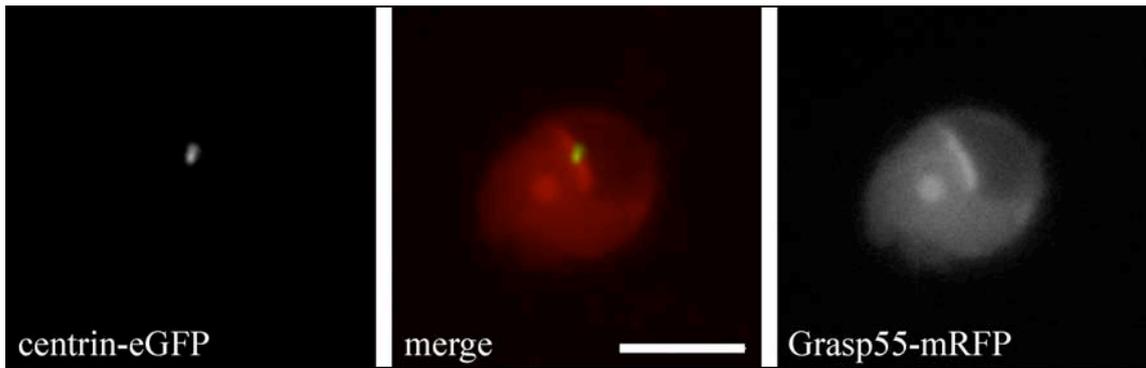
Figure 16 – Fluorescence microscopy image of Toxoplasma gondii treated with 1µM oryzalin. The parasite is not able to divide properly. The centrosomes (left and green) are duplicated but unable to move apart. The Golgi (red and right) has extended enormously but is not capable of division and separation. Note the circular artifact in the mRFP channel. Scale bar is 5µm.

3.5.4 Time lapse videomicroscopy

The ultimate goal of experiments with the double stable parasite line was to obtain video-imaging of a vacuole with parasites throughout the whole life cycle. Technical problems prevented this. Hence only time-lapse videomicroscopy could be used to follow parasites through two or three stages thereby providing further information.

Figure 17 to Figure 19 combine sets of images showing the transitions between different stages of the cell cycle.
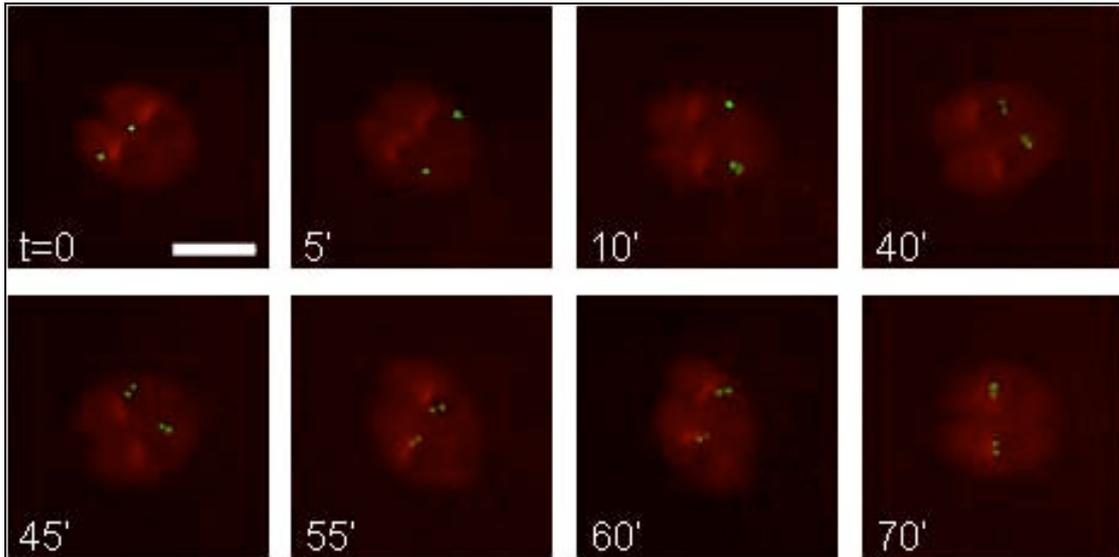
Figure 17 – Set of fluorescence microscopy images showing the transition from the end of stage 1 to stage 4 during a 70 minutes period. Scale bar is 5µm.
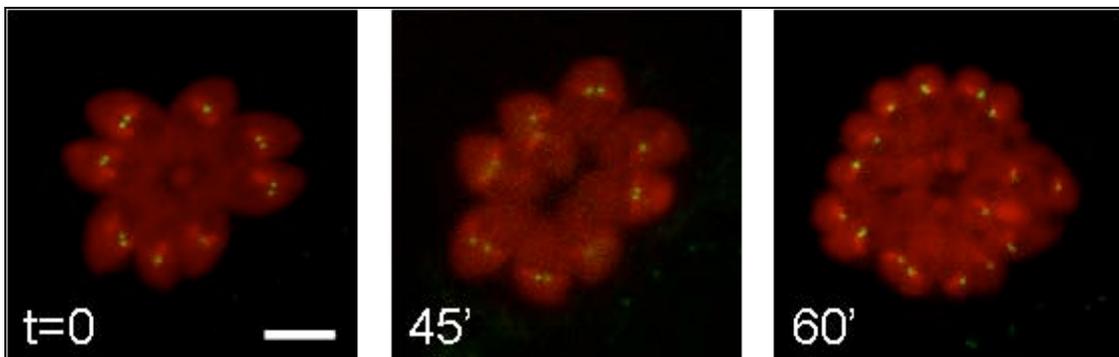

Figure 18 - Set of fluorescence microscopy images showing the transition from stage 4 to stage 1 during a one hour period. Scale bar is 5µm.
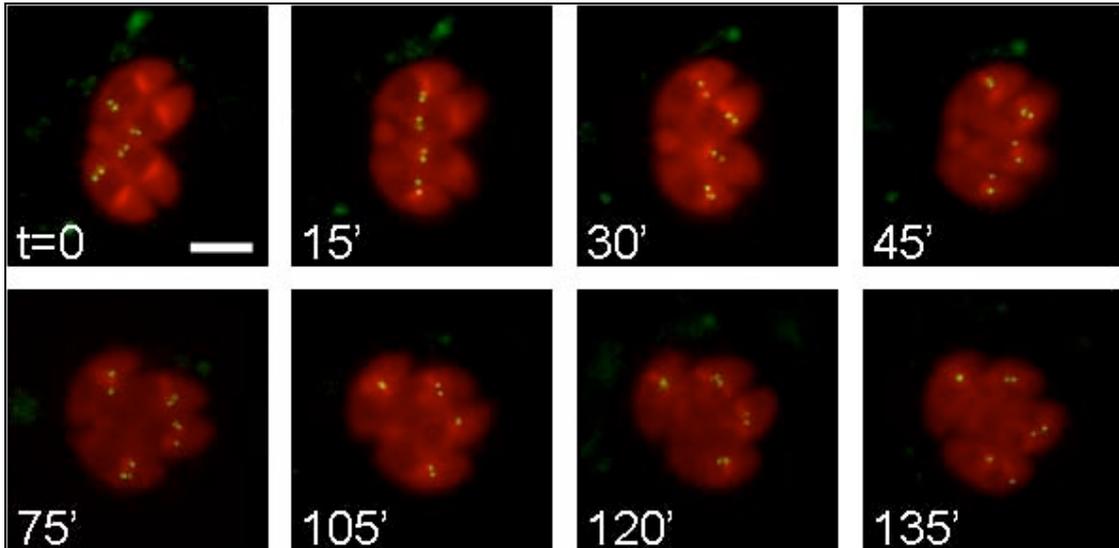
Figure 19 - Set of fluorescence microscopy images showing the transition from stage 3 to stage 1 during a 135 minutes period. Scale bar is 5µm.

The average parasite life cycle took six to eight hours. The knowledge of the relative distribution to the specific stages and the information from the time lapse videos combined allowed an estimation of the order and dynamics of events.

Immediately after mitosis, the centrosome in each daughter cell was located at one end of the Golgi and remained there for approximately 3 hours as the Golgi grew. The centrosome then detached and moved towards the basal part of the parasite, where it duplicated. Together, this took about one hour. The two centrosomes then moved back to the duplicated Golgi (another half hour), which had begun to separate, and stayed in its vicinity for about one hour. The centrosomes finally positioned themselves near the site of separation and were found at the inner ends of the newly duplicated Golgi stacks until cytokinesis was finished after about another hour, and another round of the cycle began anew.

4   Discussion

The inheritance of the Golgi apparatus during cell division is a very controversial field. There are mainly two opposing views. One regards the Golgi as an independent organelle while the other regards the Golgi simply as an extension to the ER. The reversible redistribution of Golgi material to the ER during mitosis or BFA treatment has been taken as evidence for the second model. It claims that Golgi can grow *de novo*, that is from the ER without any other Golgi. [32; 33; 78] The second model claims the Golgi to be an independent organelle, which cannot grow *de novo*, but rather needs a template presumably given by the old Golgi. This is supported by the observation, that microsurgically generated cytoplasts lacking the Golgi, cannot grow a new one. Only when treated with BFA in advance, which causes Golgi resident enzymes to spread throughout the cytosol, cytoplasts are able to build a new Golgi. [41; 50; 58]

The Golgi apparatus has very well been characterized in mammalian cells but just recently, protozoan parasites have been "discovered" as model organisms to study this organelle. [20; 51] The greatest benefit of *Toxoplasma gondii* as a model organism is its simplicity. *Toxoplasma gondii* parasites have only a very limited set of organelles and most practically, only a single-unit Golgi apparatus. [26] Mammalian cells display multiple Golgi copies, which makes it nearly impossible to follow a selected one of them. In *Toxoplasma gondii* this is possible and it allows to study some fundamental aspects of Golgi biology.

A limitation of working with *Toxoplasma gondii* is the small size of the parasites (eight by two micrometers). For light microscopic studies this means working at the physical limits of wavelength and resolution. However, the parasites are easy to grow and not very demanding to sustain. Since parasitologists have long been interested in the pathogenic effects of this organism, there is a lot of expertise and molecular tools are also available. [26; 54; 72]

In an earlier study of Golgi biogenesis in *Toxoplasma gondii* it has been shown that the Golgi apparatus grows by lateral extension and is then duplicated by medial fission, when critical size is reached. These copies divide again, transiently yielding 4 Golgi

copies that refuse to form two copies to be distributed between progeny. This shows not only that the Golgi is capable of independent replication but also poses the question of similarity to another organelle, the centrosome. [51]

A position of the Golgi near the microtubule organizing centre is characteristic of many cells. A single-copy Golgi organism allows documenting this relationship more precisely. It has previously been noticed that the Golgi apparatus in *Toxoplasma gondii* can often be found close to the centrosomes. But some of these observations have never been followed-up or published and others have not been quantified and moreover have been made in fixed images, so that nothing could be told about the dynamics of this relationship. [69]

Interestingly, just recently a study found that another *Toxoplasma gondii* organelle, the apicoplast, is divided by association with the centrosomes. The ends of the dividing apicoplast are closely linked to the centrosome. [71]

Together, these observations have raised the interest in studying the relationship of the Golgi and the centrosomes in *Toxoplasma gondii*. Taking advantage of the benefits of this model organism further information on this relationship has been obtained, implications have been derived from these observations and shall be discussed.

In the first step of this study the preliminary observations have been tested and the results quantified. Immunofluorescence techniques have been applied to visualize both Golgi and the centrosomes in fixed parasites. These studies show a spatial relation in approximately 80 percent of the parasites. Moreover the data implicate that Golgi and centrosome duplication occur around the same time, as the number of organelle copies correlates. The greater number of cases in which duplicated centrosomes but single Golgi were observed, suggests that centrosome duplication slightly precedes Golgi duplication, and is most possibly occurring when the two organelles are separated. Since these observations are made in fixed images all hypotheses about the order or kinetic of duplication events remain speculative. The results, however, are in line with the previous observations by IF (T. Stedman, Yale University, [69] and our own group, unpublished) and by EM (D.S. Roos, University of Pennsylvania, unpublished).

To gain further insight into the dynamics of events it was necessary to establish a suitable live-cell system in which both the centrosome and the Golgi can be followed

through the cell cycle. A parasite line stably expressing endogenous centrin tagged to EGFP as a centrosome marker has been kindly provided by a collaborating laboratory (D.S. Roos, University of Pennsylvania). The previously described Golgi marker, GRASP55, was tagged with mRFP as a fluorescent reporter for the Golgi. mRFP has not been used previously in *Toxoplasma gondii*, but the GRASP55mRFP construct works efficiently. The Golgi is easily detectable despite a constant homogeneous cytosolic background. The generation of a double stable parasite line expressing both constructs simultaneously provides the tools for further and deeper analysis of the relationship between both organelles. A downside of the double stable line is its sensitivity to fluorescence microscopy, leading to fading signals or cell death. This problem might arise from the overexpression of the two proteins and their reporters and the prolonged exposure to high-energy light during microscopy. These conditions were necessary to focus the microscope manually during video acquisition.

Observations in the double stable line confirm the results with fixed parasites. But beyond the fact that the centrosomes are attached to the Golgi for about 80 percent of the time, the order of events can be determined and its dynamics approximated.

There are 5 stages in the parasite's life cycle. Immediately after mitosis, the centrosome in each daughter cell is located at one end of the Golgi stack and remains there as the Golgi grows. (stage 1) The centrosome then detaches and moves towards the basal part of the parasite (stage 2), where it duplicates. (stage 3) The two centrosomes move back to the duplicated Golgi, which has begun to separate. (stage 4) The centrosomes then position themselves near the site of separation and are found at the inner ends of the newly-duplicated Golgi stacks most of the time, until the end of cytokinesis and the beginning of a new cycle. (stage 5)
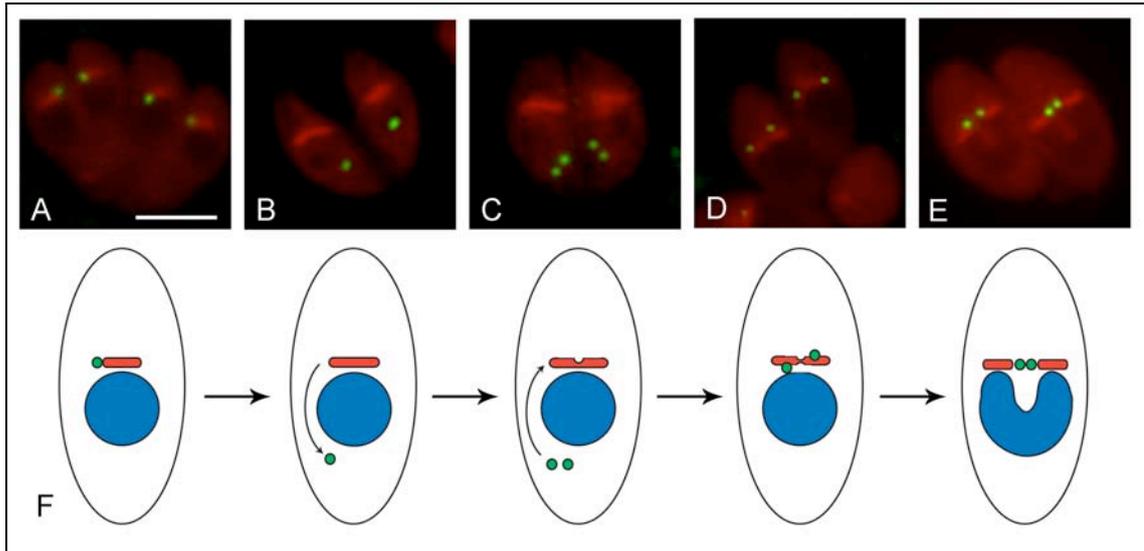
Figure 20 – Five stages in the *Toxoplasma gondii* cell cycle. Representative fluorescence microscopy images of parasites in the five different stages (A-E) are shown along with a schematic (F). Scale bar is 5µm.

The distribution of parasites to the stages has been evaluated. Combined with the information from partial cell cycle clips a model of the Golgi and centrosome cycles in *Toxoplasma gondii* has been developed.
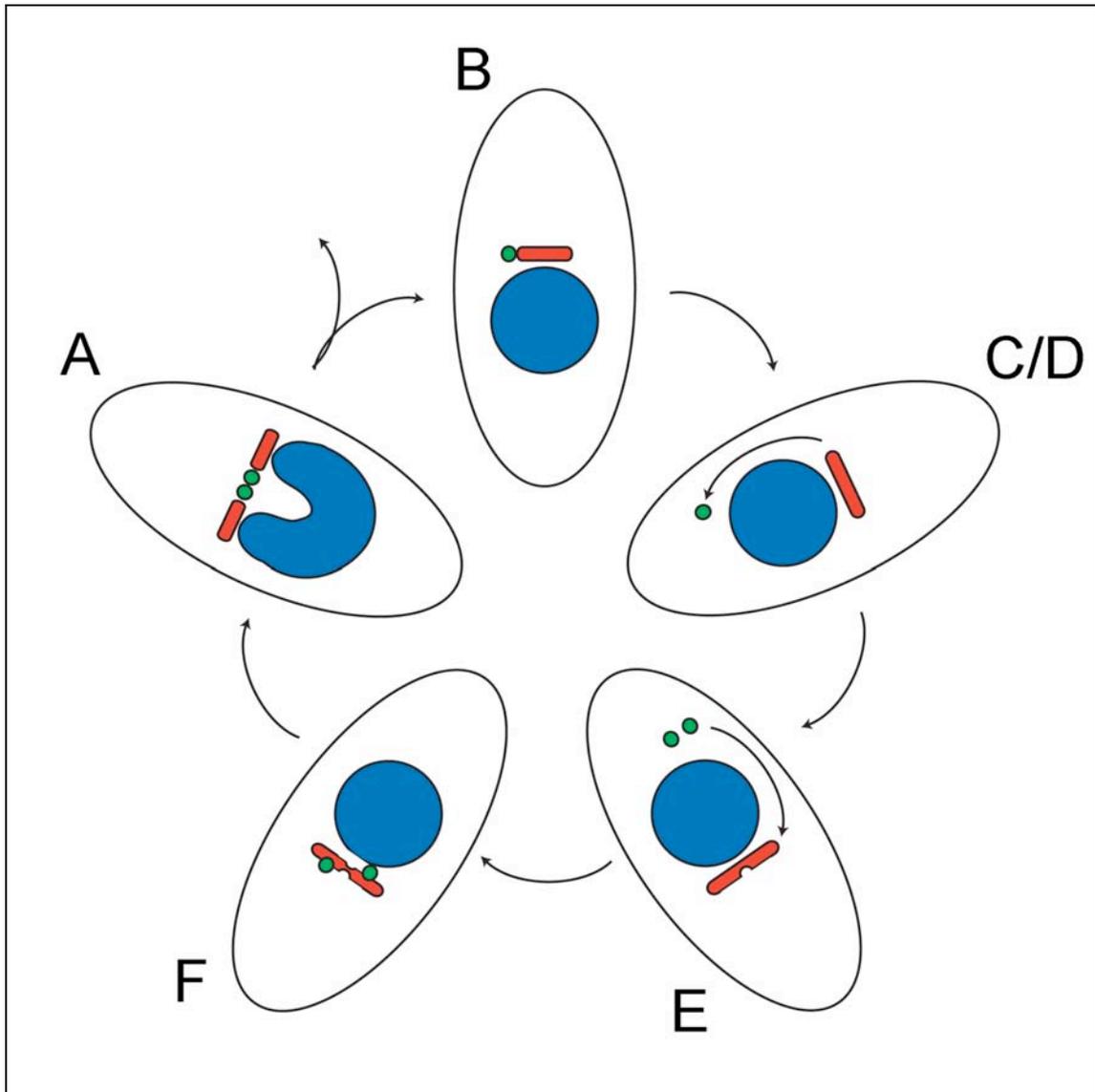
Figure 21 – *Toxoplasma gondii* cell cycle model. This modell is based upon the observations in fixed as well as living parasites. Letters correlate to the stages in Figure 14

The results from this study and the model derived argue for a close spatial relationship of Golgi and centrioles in *Toxoplasma gondii*. Due to the descriptive approach no functional assertion can be made. But the close physical relation suggests a functional hypothesis.

Centrosome attachment to one side of the Golgi in *Toxoplasma gondii* resembles the situation in mammalian cells, where this pericentriolar position of the Golgi apparatus has been explained by its prime position in protein sorting and transport. [53] The same applies to *Toxoplasma gondii*. But the proximity of the centrosome and the single-copy

Golgi goes beyond the relationship in mammalian cells. It seems as if the centrosome is located to the Golgi to ensure optimal transport of membrane material to this organelle. This correlates with the description of lateral extension occuring at this time. Perhaps membrane transport to the MTOC leads to addition of membrane material to exactly that part of the Golgi that is localized next to the centrosome. This hypothesis can be tested by photobleaching experiments to show that the lateral extension is due to unilateral addition of membrane material. Preliminary results seem to confirm this, but size and bleaching effects limited these experiments, so that the results are not conclusive. It is however an interesting approach to follow up, once the experimental conditions have been improved.

The movement of the centrosome to the basal part of the parasite is perhaps the most striking observation made in this study and has not previously been reported. The process of centrosomal duplication / separation only occurs once it has moved away from the Golgi. It may be that duplication / separation is incompatible with this association or requires a different environment (like the ER, which is mostly located in the basal part). Alternatively, it may be that another organelle, in addition to the Golgi, apicoplast and nucleus, also needs the duplicating (or duplicated) centrosomes for its division and is located in the basal part of the cell. Further studies will be needed to investigate the life cycle of other organelles.

The downward movement of the centrosomes shown in our results was not described in a previous study focusing on the apicoplast division by association with the centrosomes. [71] This study used fixed cells, which might explain how this stage has been missed, or misinterpreted.

The idea that the centrosomes might play an essential role in the fission process of Golgi duplication is contradicted by the fact that early signs of medial fission can already be observed while the centrosomes are still at the basal part.

Immediately prior to mitosis, Golgi and centrosomes line up in a very distinct manner. The centrosomes are found on the inside of the duplicated Golgi. During cytokinesis the centrosomes seem to move the Golgi apart. This is analogous to the apicoplast study mentioned above. In contrast to the apicoplast, the Golgi seems to be pushed rather than pulled. Centrosome function might be essential for proper Golgi separation. This

hypothesis has been tested by a simple drug experiment. Cells have been treated with the microtubule-disrupting agent, oryzalin, at a level known to prevent chromosomal segregation. [46] Cells are thereby arrested in a state where duplicated centrosomes are found in the middle of an elongated Golgi incapable of separation. This is in support of an essential role of the centrosomes or at least the microtubular system in proper Golgi division.

Another implication of these results is the possible use of oryzalin as an anti-parasitic agent. Oryzalin is a dinitroaniline herbicide affecting microtubules in plants and protozoa such as *Toxoplasma gondii*, but not in vertebrate or fungal microtubules. Moreover, it could be shown in-vitro that oryzalin does not affect host cells at the concentrations sufficient to block *Toxoplasma gondii* replication. [46; 60; 70] This raises the possibility to treat the pathogen with this, or similar, drugs in animals and humans.

Work on another protozoan parasite, *Trypanosoma brucei*, and also in budding yeast argue for *de novo* biogenesis of the Golgi where the new Golgi is found at a place distinct from the existing one. [5; 20] Previous work in *Toxoplasma gondii* argues for templateted biogenesis by lateral growth and medial fission. [51] This view is supported by the findings from this thesis. The data also support an essential involvement of the centrosomes both in the separation process and in the process of lateral extension.

The linkage between microtubules and membrane bound organelles is a promising field of study. Various linker proteins have been described in mammalian cells, and it has been shown that this link is essential for Golgi integrity. [8; 23; 53] While this study cannot provide functional or mechanistic evidence the morphological findings, together with the results from selective microtubule disruption in the oryzalin drug experiment, support this hypothesis. Combined with the simplicity of the organism, this suggests that *Toxoplasma gondii* is a useful model in which to study further questions of organelle biogenesis.

5   Summary

Protozoan parasites have been used as model organisms, to study Golgi biogenesis. Whereas observations in *Trypanosoma brucei* argue for *de novo* formation in a spot distinct from the existing Golgi, the results in *Toxoplasma gondii* suggest biogenesis by growth through lateral extension and medial fission.

This work focuses on the role of the centrosomes in this process in *Toxoplasma gondii*. Fixed and antibody stained parasites show a close spatial relationship of the Golgi and the centrosomes in approximately 80 percent of the time.

A cell line expressing a centrosome marker tagged to EGFP has been obtained. A construct linking a Golgi marker protein to mRFP has been produced and verified.

The generation of a parasite line that stably expresses the centrosome as well as the Golgi marker, both tagged to different fluorescent reporters, yields further insight into the relationship of both organelles and its choreography. A model for the Golgi and centrosome cycles in this parasite is proposed. It shows that the centrosome is attached to the Golgi as it grows by lateral extension. The centrosomes then move to the basal part of the parasite to duplicate. Having moved back to the meanwhile duplicated Golgi the centrosomes position themselves in the middle of the two and seem to push them apart during cytokinesis.

The microtubular action is thought to be essential for this, since drug interference with oryzalin, a micro-tubule disruptive agent in the parasite, leads to a failure of both centrosome and Golgi separation. Therefore this drug is proposed as a possible anti-parasitic agent.

The morphological observations combined with the result of the drug experiment are promising and suggest to further study the functional relationship of both organelles. This might yield intersting results with implications for other protozoan organisms and mammalian cells as well.

6   Zusammenfassung

Protozoen dienen als Modelorganismen für die Erforschung der Biogenese des Golgi-Apparates. Während Beobachtungen in *Trypanosoma brucei* für eine *de novo* Bildung entfernt vom alten Golgi sprechen, deuten die Ergebnisse in *Toxoplasma gondii* darauf hin, dass der neue Golgi-Apparat durch laterales Wachstum und Abtrennung vom alten entsteht. Zielsetzung dieser Arbeit ist es, die Rolle der Zentrosomen in diesem Prozess zu beschreiben.

In fixierten und antikörper-markierten Parasiten zeigt sich eine sehr enge räumliche Beziehung von Golgi-Apparat und Zentrosomen. Eine Parasiten-Zellinie, die einen an EGFP gebundenen Zentrosom-Marker exprimiert, wurde beschafft. Ein anderes Konstrukt, das ein Golgi-Protein mit mRFP verknüpft, wurde gentechnisch hergestellt und als geeigneter Golgi-Marker in den Parasiten bestätigt.

Die Herstellung einer Zellinie, die gleichzeitig und stabil sowohl den Zentrosom- als auch den Golgi-Marker exprimiert, läßt weitergehende Erkenntnisse über die Beziehung der beiden Organellen zu. Daraus wird ein Modell für die Golgi- und Zentrosomzyklen in den Parasiten abgeleitet: Das Zentrosom ist an den Golgi angeheftet, während dieser wächst. Das Zentrosom wandert dann zur basalen Seite des Parasiten, um sich dort zu teilen. Nachdem sich die Zentrosomen geteilt haben, kehren sie zum Golgi-Apparat zurück, der mittlerweile auch geteilt ist. Sie plazieren sich zwischen den beiden Golgi-Apparaten und scheinen diese während der Zytokinese auseinander zu schieben.

Hierzu bedarf es der Mitarbeit der Mikrotubuli. Unter der Gabe von Oryzalin, einer die Mikrotubuli zerstörenden Substanz, kommt es zu einer fehlerhaften Teilung von Zentrosomen und Golgi-Apparat. Daher wird dieses Mittel als mögliches Therapeutikum bei Toxoplasmose vorgeschlagen.

Die morphologischen Beobachtung zusammen mit den Ergebnissen des pharmakologischen Experiments sind vielversprechend. Die Beziehung beider Organellen sollte auch auf funktioneller Ebene weiter untersucht werden. Daraus könnten sich interessante Resultate ergeben, die ebenso Implikationen für andere Protozoen wie z.B. *Plasmodium falciparum* als auch für Säugetierzellen haben könnten.

7   Literature

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8   Acknowledgements

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Bücher

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Spanisch   fließend in Wort und Schrift

Französisch   Grundkenntnisse

Latein   Großes Latinum