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Review

New insights regarding the biology of *Giardia lamblia*

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Abstract

Giardia lamblia is one of the most common causes of intestinal disease in humans. To adapt to environments both inside and outside of the host's small intestine, this protozoan parasite undergoes significant developmental changes during its life cycle. In this review, we analyze and discuss the most recent findings regarding the process of *Giardia* trophozoites differentiation into infective cysts as well as the mechanism of antigenic variation, which allows the parasite to cause chronic and recurrent infections in susceptible individuals.

Keywords: Parasite; Cell differentiation; Cyst; Protein trafficking; Antigenic variation

1. Introduction

Giardia lamblia, also called *Giardia intestinalis* or *Giardia duodenalis*, is a single-celled organism that inhabits in the upper small intestine of humans and several other vertebrates [1]. This parasite infects thousands of people all over the world and is the cause of Giardiasis, an infection characterized by asymptomatic presentation or acute and chronic manifestations including diarrhea and malabsorption [1]. *Giardia* infections are initiated by ingestion of cysts from contaminated water or food, or through direct fecal-oral contact [2]. Cysts excyst in the upper small intestine releasing the trophozoites, which proliferate while attached to the epithelial cells of the gut [2]. The parasite is non-invasive, and neither toxin nor virulence factors have been clearly defined [1]. *Giardia* posses two structurally and biochemically different developmental stages: the motile, flagellate trophozoites that are responsible for the clinical manifestation of the disease, and the cyst forms, the infective stage of the microorganism, in which trophozoites are surrounded by a rigid, protective cyst wall that allows the parasite to survive outside the host's intestine [1] (Fig. 1).

Giardia is simple with regard to structure. The parasite possesses some basic characteristics of eukaryotic cells such as the presence of two nuclei with nuclear envelopes linked to the endoplasmic reticulum (ER), a very complex cytoskeleton and lysosome-like peripheral vacuoles (PVs) that are located underneath the plasma membrane. On the other hand, *Giardia* lacks organelles typical of higher eukaryotes, like classical mitochondria, peroxisomes, and a morphologically evident Golgi apparatus [1]. *Giardia* are considered a primitive eukaryote because phylogenetic studies of several genes showed that this organism belongs to the earliest diverging branch of the eukaryotic lineage [3], even though they have some of the metabolic pathways typical of prokaryotic organisms [1]. However, the lack of some eukaryotic features might have been secondarily lost due to its parasitic life style and metabolic pathways could be acquired by lateral gene transfer [4,5].

Giardia possesses two mechanisms of adaptation to survive inside and outside the host intestine, antigenic variation and encystation, respectively [2]. Antigenic variation consists of the continuous switching of specific surface antigens thought to be important for evasion of the host's immune system [6]. Encystation, on the other hand, comprises the formation of a resistant cyst wall that permits the parasite to survive under

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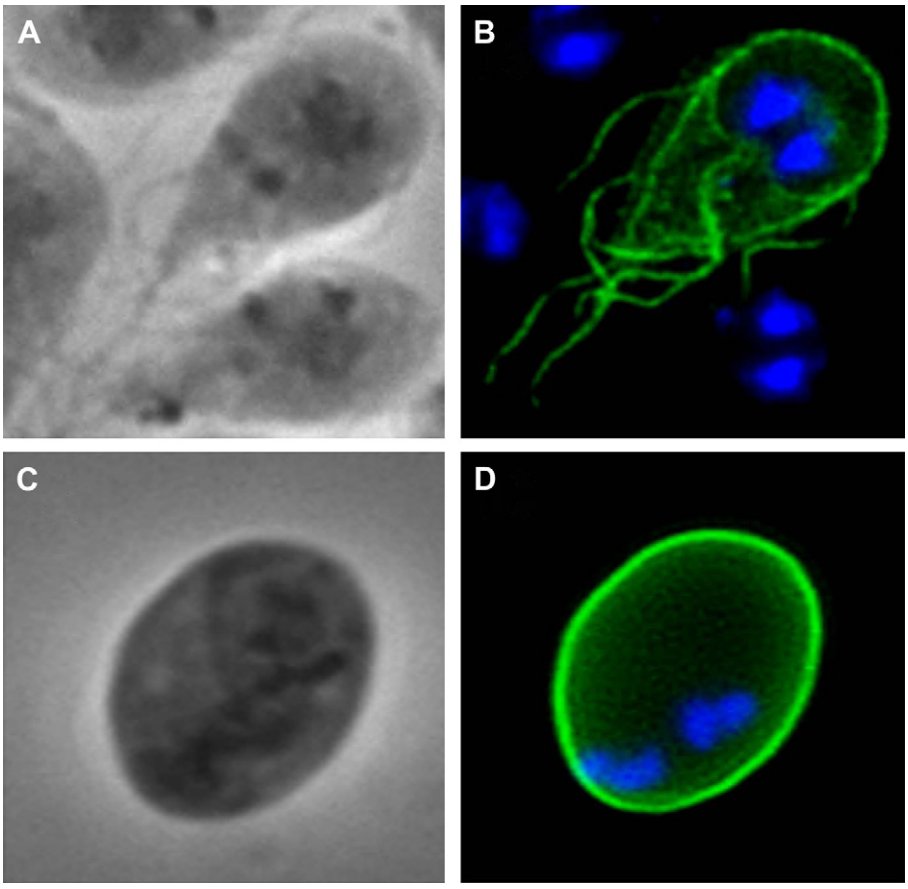

Fig. 1. Morphology of *Giardia lamblia*. A: Phase contrast image of *Giardia* trophozoites; B: Immunofluorescence image of *Giardia* trophozoites labeled with a monoclonal antibody against VSP 9B10 (green), nuclei are stained with DAPI (blue), in which variable expression of a given surface protein can be observed. C: Phase contrast of a *Giardia* cyst; D: Immunofluorescence of *Giardia lamblia* cyst labeled with an antibody specific for CWP 2 (green), nuclei staining with DAPI (blue).

hostile external environmental conditions and guarantees the transmission of the infection to susceptible hosts [2] (Fig. 1).

In this review, we describe and discuss the latest most relevant findings about the biology of this parasite and introduce new questions that are of significance to understand key molecular processes in this fascinating protozoan parasite.

2. Structural characteristics of *Giardia*

The vegetative trophozoite is a pear-shape cell about 12–15 μm long, 5–7 μm wide, and 1–2 μm thick. *Giardia* possesses two nuclei surrounded by nuclear envelopes that remain practically intact throughout the cell cycle [1,7]. *Giardia* has a complex cytoskeleton that maintains the shape of the parasite and anchors the four pairs of flagella, which emerge ventrally, anteriolaterally, posteriolaterally, and caudally from the cell body; the median body; and the ventral disk [8]. These structures are important for attachment of the trophozoites to the enterocytes in the intestine [2,8,9] and are the target of some drugs currently in the market used to treat the infection [9].

Giardia has a well-developed ER and can form secretory vesicles [10,11], some of which are clearly visible during encystation (the encystation-specific secretory vesicles or ESVs), and which transport cyst wall components to the cell surface for assembly into the extracellular cyst wall [2]. Surprising, a typical Golgi apparatus comprising a set of flattened cisternae is not found at any time during the *Giardia* life cycle [10]. *Giardia* also lacks "typical" eukaryotic organelles, like peroxisomes and mitochondria, although a mitochondrial remnant has been described and characterized recently [12]. In the cell cytoplasm and adjacent to the plasma membrane (except in the region of the ventral disk), there are numerous vesicles and tubules, the PVs, that function both as endosomes and lysosomes [10].

The formation of the cyst allows the parasite to survive outside the host and to resist the adverse conditions that the parasite confronts when cysts are released with the feces of infected individuals into the environment [1,2]. Therefore, the cysts are responsible for the transmission of the parasite among susceptible hosts [1,2]. *Giardia* cysts are oval in shape and range in size from 6 to 10 μm. The cyst wall varies from 0.3 to 0.5 μm in thickness that is formed by an outer filamentous layer and an inner membranous layer including two membranes that enclose the periplasmic space [1]. The biochemical composition of the cyst wall is composed of carbohydrates, in the form of N-acetyl galactosamine polymers [2], and cyst wall proteins (CWPs) [13,14]. The

cytoplasm of the cyst contains two or four nuclei depending on the stage of maturation, the contracted flagella, and fragmented portions of the ventral disk [2,9].

Giardia trophozoites have four pairs of flagella composed of microtubules in a typically eukaryotic $9+2$ arrangement that are built from basal bodies between both nuclei [8]. The flagella are important for parasite motility, during the excystation process (see below), and may function (the ventral pair) in the attachment of the parasite to the epithelial surface by creating negative pressure under the ventral disk [15]. The basal bodies also are involved in nuclear division (see below) [7,16,17]. The funis is a structure made of short arrays of microtubules that are associated dorsal and ventrally with the axonemes of the caudal flagella. The median bodies are formed by an irregular set of microtubules and have an unknown function. In general, median bodies have a comma-shape structure that varies in size and thickness, and are located transversally, perpendicular to the central axis. It has been proposed that the median bodies function in nucleation or reserve of microtubules and in the biogenesis of the ventral disk [18].

The ventral disk is a unique structure of this genus and covers the anterior half of the ventral side of the trophozoite. It is linked to the plasma membrane by short fibers [8] that are composed of α- and β-tubulin, contractile proteins, and cytoskeletal proteins called giardins [9]. In addition to its function as a sucking disk that allows the parasite to attach to the epithelial microvillus of the upper small intestine, this structure also has been implicated to play roles in nuclear division [7,16]. Parasites do not invade the host epithelium, so the establishment of *Giardia* infections depends on the ability of the trophozoite to attach to the intestinal wall and the ventral disk is a key organelle in this process. This structure undergoes profound changes during the process of differentiation into cysts where it disassembles and is stored as fragments during cyst formation [9]. During excystation, the newly tetranucleated trophozoites enter rapidly into cytokinesis and reassemble the disk fragments into two new adhesive disks [9].

Giardia trophozoites possesses two diploid ($2 \times 2N$), oval-shaped nuclei, of about 1 μm in diameter each, located in the anterior half of the cell, at the left and right sides of the longitudinal axis and divide by binary fission [7]. Apparently, both nuclei contain the same genetic information [19], are transcriptionally active [19,20], divide at the same time [15], and are equally partitioned at cytokinesis [20]. On the other hand, the mature cyst is tetranucleated, which is due to a nuclear division without cytokinesis occurring during the last phase of encystation, thereby generating cells with a DNA content of 16N ($4 \times 4N$) [21]. During excystation, cytokinesis occurs producing two trophozoites with 8N ($2 \times 4N$) each, follow by a complete cell division that generates four trophozoites containing 4N ($2 \times 2N$) each [21]. An important question is how *Giardia* coordinates its cell division to produce two identical daughter nuclei. Recently, some controversial findings in this regard have been reported, although all authors agree with the fact that the nuclear envelope remains almost intact during the entire cell cycle of the parasite [7,16,17].

A series of electron microscopic studies proposes that the ventral disk and the basal bodies of the flagella command the mitosis of *G. lamblia* [7,16]. These authors did not observe microtubular spindles either inside or outside the nuclei during the mitotic process. Conversely, a more recent report describes the presence of a microtubule spindle during karyokinesis [17]. These authors used molecular markers, such as anti-tubulin antibodies to label the microtubule spindles, an anti-centrin antibody to label the centrosomes (this protein is found in the basal bodies and centrioles in other organisms) and CenH3 to mark the centromere, which is a variant histone H3 that collaborates with the kinetochore structural assembly [22]. The authors postulate that *Giardia* exhibits semi-open mitosis, where two nuclear microtubule spindles enter into each nucleus through a polar opening in the nuclear envelope and supervise chromosome segregation, interacting with the kinetochores (marked with CenH3-GFP). Apparently, the basal bodies act like centrosomes (marked with anti-centrin antibody) collaborating with this process. The nuclear envelope remains intact during the entire process, and the chromosomes are segregated along the left-right axis and cytokinesis occurs along the longitudinal axis. This is an elegant description of the cell division process; however, in this study, the authors used cells transfected with GFP-CenH3. GFP is not fluorescent in *Giardia* because they are almost completely anaerobic organisms, so GFP is only used as a tag in static experiments. CenH3 is a key molecule in kinetocore assembly with a molecular weight of about 17 kDa (GFP is larger with about 27 kDa); therefore, this tag could interfere in the interpretation of the result. Due to these discrepancies, it is necessary to determine the dynamics of nuclear division in *Giardia* once new tools become available.

In *Giardia*, the heterozygosity of its genome is less than 0.01% [3]. This is an unsuspected characteristic because the parasite possesses two nuclei and no sexual stages have been described; however, some genes homologous to those of the meiotic process in higher eukaryotes are present in *Giardia* [3]. A recent report describes a possible mechanism that maintains low-grade heterozygosity, which includes karyogamy and exchange of genetic material between the nuclei. By using episomal plasmid probes, tagging of giardial homologs of meiosis-specific genes and transmission electron microscopy (TEM), these authors showed fusion of the nuclei (or karyogamy) in cysts, and subsequently, somatic homolog recombination facilitated by *Giardia* meiosis components [23].

Like in higher eukaryotes, *Giardia* nuclei are surrounded by the nuclear envelope, formed by inner and outer membranes that are maintained during the cell cycle, and are perforated by nuclear pore complexes. The outer membrane is studded with a few ribosomes; but during encystation their numbers increase, which correlates with the higher rates of transcription and translation during this developmental change in trophozoites [24]. This membrane is contiguous with the ER system. In ultrastructural observations, the inner membranes of *Giardia* nuclei are covered by a dense material, probably the nuclear lamina, which is known to provide structural support to the nuclear envelope [24]; however, immunocytochemistry using an

anti-mammalian lamin antibody did not detect this molecule in the nuclei of the parasite, and its gene is not present in the *Giardia* genome [24]. The structure of the nuclear pore complex is similar to those found in other eukaryotic cells, with a different pore number in an asymmetric distribution in each nucleus. This observation may reflect a distinct metabolic activity, which contrasts with the previous suggestion that both nuclei are equivalent [19].

In higher eukaryotes, more than 50 different proteins, called nucleoporins, have been identified, which are arranged with a striking octagonal symmetry. In *Giardia*, the molecular composition of nuclear pores is completely unknown.

The nucleolus is a specialized region of the nucleus where rRNA synthesis and processing as well as further ribosome subunit assembly take place. It previously was suggested that the rDNA of *Giardia* does not form the nucleolar organizing region (NOR) structure and that it may represent a primitive state of this cell [25]. A recent report [26], however, using nucleolar-specific molecular markers (fibrillarin and rRNA-pseudouridine synthase, CBF5), show at the light, fluorescence, and TEM levels, the presence of 0.2–0.5 μm-sized intra-nuclear sub-compartment domains of fibro-granular nature at the anterior zone of both nuclei that may well correlate with nucleolar organizing regions in *Giardia* trophozoites.

More recently, novel approaches and techniques have provided interesting information regarding many aspects that were controversial about the structural characteristics of this parasite. Nevertheless, we are far from fully understanding how the molecular discoveries (greatly helped by the sequencing of the entire *Giardia* genome) are linked to the structural observations reported about the parasite.

3. Molecular mechanisms of *Giardia* differentiation into cysts

The study of cell differentiation in several important human parasites, such us *Entamoeba*, *Cryotosporidium*, and *Microsporidium*, among others, is difficult because the stimuli that trigger differentiation in these microorganisms are still unknown. Therefore, *Giardia* has become an interesting model system to study the mechanism of cellular differentiation in early branching eukaryotes [2,27].

Encystation of *G. lamblia* can be divided into three stages [2]. The first is the reception of the stimulus for encystation, the transmission of this signal to the nuclei, and the expression of encystation-specific genes. The second step comprises the synthesis of precursors and cyst wall molecules, the biogenesis of secretory organelles absent in non-encysting trophozoites, and the intracellular trafficking of the cyst wall components. Finally, the last stage of encystation includes exocytosis of ESVs and assembly of the extracellular cyst wall (Fig. 1).

The ability to induce encystation of *G. lamblia in vitro* was described more than 20 years ago [28], but the specific and unique stimuli that trigger the process were discovered ten years later [29]. The capacity to reproduce cyst formation in the laboratory allowed the identification of molecules that are specifically induced during encystation, including components of the ESVs and the cyst wall, particularly CWP-encoding genes, which, at the same time, permitted a better understanding of the molecular basis and regulation of the encystation process [2].

Cholesterol deprivation was demonstrated to be necessary and sufficient to trigger differentiation of *Giardia* trophozoites into cysts [29]. This parasite cannot synthesize cholesterol *de novo* and trophozoites take up this lipid from the milieu of the upper small intestine [1]. The host intestine absorbs this lipid at the jejunum and below this intestinal region; the concentration of cholesterol is very low. When *Giardia* travel down the intestine, the host detects this condition and triggers the encystation process [2]. Previously, it was demonstrated that bile salts also were capable of inducing encystation, but it later was shown that bile salts can form stable micelles with cholesterol, inhibiting its uptake by the cells [2]. Nevertheless, how *Giardia* detects low levels of cholesterol remains unknown.

3.1. Gene regulation during encystation

In mammalian cells, a family of membrane-bound transcription factors located at the ER, called the Sterol Regulatory Element Binding Proteins (SREBPs), plays key roles in the regulation of cholesterol metabolism [30]. Low levels of cholesterol alter the fluidity of the plasma membrane and, in collaboration with others molecules, produce the cleavage of the cytoplasmic domain of SREBP, which then interacts with the SRE present in the promoter region of sterol regulated genes to up-regulate transcription [31]. Since no SREBP has been found in the *Giardia* genome, a report described evidence of a similar process in *G. lamblia* in a particularly indirect manner. The authors showed that when transfecting mammalian cells with vectors containing the promoter sequence of the CWP2 gene fused to the luciferase reporter and then depleting cholesterol from the culture medium, the reporter gene expression increased 2- to 3-fold [31]. Unfortunately, the authors did not show that a similar increase in expression occurs for CWP1, CWP3, and other genes that are known to increase their expression during encystation. Moreover, the expression of CWP2 is known to increase near 140-fold during *Giardia* encystation [13], in contrast to the 2- to 3-fold increase of the CWP2 promoter-luciferase construct [31]. Therefore, the mechanism by which cholesterol deprivation triggers specific gene regulation remains enigmatic. We support the hypothesis that changes in the fluidity of the *Giardia* membranes due to the lack of cholesterol may modify the activity of membrane-anchored enzymes, which in turn trigger a signal transduction pathway that ends with the transcriptional activation of encystation-specific genes [13].

To date, gene regulation during encystation is poorly understood. Several encystation-specific genes have been identified and characterized during the last decade, and shown to be up-regulated with similar kinetics during encystation, suggesting that their regulation is at the transcriptional level [13]. The genome of *Giardia* is compact in structure and

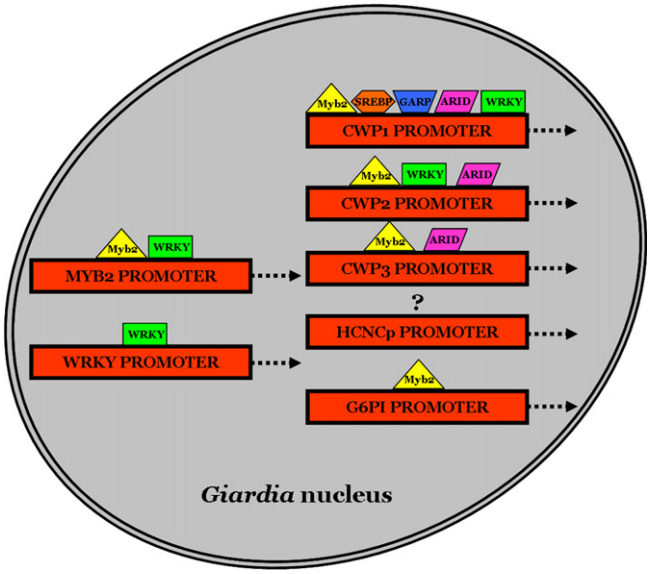

Fig. 2. Schematic representation of putative transcription factors described to be involved in encystation of *Giardia*. *CWP1*: Cyst Wall Protein 1; *CWP2*: Cyst Wall Protein 2; *CWP3*: Cyst Wall Protein 3. *HCNP*: High-Cysteine Non-VSP Cyst protein; *G6PI*: Glucose-6-phosphate isomerase; *Myb2*: *myb*-related transcription factors; *SREBPs*: Sterol Regulatory Element Binding Proteins; *GARP*: Golden Arabidopsis Response Regulator Protein; *ARID*: AT-rich interaction domain; *WRKY*: wrky transcription factor.

contains 11.7 Mb divided into five chromosomes [3]. DNA synthesis and transcription are very simple and many characteristics of transcription seem to be uncommon [3]. Several genes overlap and others are within 100 nucleotides of an adjacent ORF [3]. *Giardia* has a highly divergent TATA-binding protein, lack 8 out of 12 general transcription factors, and neither TATA nor CCAAT boxes have been described [32]. Apparently, AT-rich sequences can act as initiators of transcription for many genes. In the promoter of CWP2, a positive *cis*-acting element (−23 to −10) and a negative *cis*-acting element (−64 to −52) have been postulated to exist [33], and, recently, several publications described putative transcription factors that regulate the expression of encystation-specific genes (summarized in Fig. 2):

MYB-like transcription factors: Myb2 was the first putative giardial transcription factor to be identified and the first associated with up-regulation of encystation gene expression. Myb family members are important transcription factors in fungi, plants and mammals, regulating developmental processes as well as the cell cycle. Two putative Myb genes in *Giardia* contain the typical *myb* domain. *Giardia myb2* is induced during encystation, whereas the expression of *myb1* is constitutive. Epitope-tagged Myb2 was localized to both nuclei of the parasite. Myb2 interacts with double-stranded DNA and binds specifically to a C(T/A)ACAG motif, which is similar to the target of eukaryotic c-Myb, PyAACNG. This domain is present in the 5′-flanking regions of four encystation-induced genes, *cwp1*, *cwp2*, *cwp3*, *g6pi-b* and *myb2* itself [34]. Unfortunately, although the techniques are available, the expression of Myb2 was not disrupted by those authors to demonstrate a direct link between this putative transcription factor and the regulation of encystation in *Giardia*. Nevertheless, Myb2 is the transcription factor with the highest possibility to function during encystation.

ARID-like proteins (AT-rich interaction domain): ARID proteins integrate a large family of transcription factors in higher eukaryotes that regulate cell proliferation, development and differentiation. Sun's group reported that by BLAST analysis they identified two putative sequences with ARID domains in the *Giardia* genome, ARID1 and 2 [35]. Recombinant ARID1 specifically binds to the CWP1 promoter. Epitope-tagged ARID1 was found to localize to the nuclei during vegetative growth and encystation. The mRNA levels of ARID1 decrease slightly during early encystation and significantly at 24 h. The authors presented evidence from experiments where they compared the increase of the ARID1 and clearly did not show a significant enhancement in expression, as their conclusions stated. Rather, they suggested that ARID1 might function as a transcription factor because they expressed a recombinant protein and performed gel shift experiments that showed DNA-binding capacity of the molecule [35]. However, the discrepancy between what is stated in this report and what is shown is troubling. Also in this case, the putative function of ARID1 as a transcription factor for encystation remains controversial.

GARP-like proteins: This is a large family of DNA-binding proteins from plants that are involved in regulation of transcription, phosphotransfer signaling, and differentiation [36]. The authors identified four GARP-like genes named GLP1–4, which contain putative GARP domains. GLP1 mRNA levels increase slightly during encystation but GLP2, GLP3 and GLP4 were down-regulated during this process. Epitope-tagged GLP1 localized in both nuclei and the signal level was increased 10-fold during encystation. Recombinant GLP1 and GLP2 specifically bind to CWP1 promoters and to the constitutively expressed RAN gene promoter [36]. Here again, the value of GARP-1 as a transcription factor exclusive for encystation-specific genes is debatable.

WRKY-like proteins: WRKY proteins constitute a large family of DNA-binding proteins present in plants that are involved in disease resistance, stress response, dormancy and development [37]. Recently, Sun's group described a putative homolog of WRKY in *G. lamblia* (ORF: 9237) characterized by two WRKY-like domains and a Zn-finger motif, which is up-regulated during encystation [37]. Epitope-tagged WRKY was found in the cytoplasm and nuclei of vegetative trophozoites and mainly in the nuclei during encystation. Chromatin immunoprecipitation assays confirmed that WRKY binds to the 5′ upstream region of CWP1, CWP2 and Myb2 genes as well as to its own promoter. It also was shown that when overexpressing ERK1, a member of the MAPK family (see below), the WRKY mRNA levels increase, suggesting that this protein plays a role in the MAPK/ERK1 pathway. Although these results are quite interesting, there are some problems with the design of the experiments and the conclusion taken from the results. For example, a significant increase in expression of this molecule during encystation is difficult to observe, as

described by the authors. In addition, there are differences in the molecular mass of several constructs designed to determine the DNA-binding site as compared to the mass predicted by their primary sequence or by the mass of the recombinant proteins.

The same authors also reported that both antibiotics routinely used to select for cells containing the plasmids utilized to express foreign genes or tagged-variants in *Giardia* (neomycin and puromycin) are, *per se*, able to modify gene expression in this parasite [38]. In this report, this group adds more controversy to the field of gene regulation in *Giardia* because these antibiotics are commonly used by many groups without finding any relevant change in the behavior of the cell and leaves us without the information needed to understand this process. What is clear is that genes that are up-regulated during encystation are present on different chromosomes and their transcription is regulated. Therefore, transcription factors should be necessary to control the expression of these genes during differentiation [13]. Unfortunately, the results presented so far in this regard have not solved the puzzle of how this process is regulated and coordinated. Since transcription in *Giardia* has been reported to be very relaxed [39], we favor the idea that a transcriptional repressor is working to block the expression of encystation-specific genes, which should be released from the DNA at the time of differentiation.

3.2. Signal transduction pathways during encystation

The genome of *G. lamblia* presents an extensive number of putative protein kinases, which could reflex the complex requirement of signal transduction to coordinate not only cell proliferation but also the encystation–excystation processes [3]. However, the proteins and pathways involved in signal transduction have only begun to be understood. Some reports describe a possible mechanism:

ERK1 and ERK2 (Extracellular regulated kinases 1 and 2): These enzymes possess homology to members of the Mitogen-activated Protein Kinase (MAPK) family, involved in signaling in higher eukaryotes [40]. Furthermore, these proteins are activated by phosphorylation, are able to translocate to the nuclei, and phosphorylate other *Giardia* proteins, including putative transcription factors. In *Giardia*, these enzymes localize to different structures, gERK1 to the median body, ventral disc, and basal bodies, and gERK2 to the nuclei and caudal flagella. ERK2 changes its localization pattern during encystation, being more punctuate and mostly cytoplasmic.

PP2A-C (Protein phosphatase 2A): This is a highly conserved serine/threonine protein phosphatase associated with encystation and excystation processes [41]. PP2A-C localizes to cytoskeleton structures and occasionally to the cyst wall. Western blot analyses showed reproducibly that the protein level of gPP2A-C resembles mRNA expression and is up-regulated in cysts and in the beginning of the excystation process. The knock-down of this kinase produces a reduction in the number of ESVs and a significant reduction in the number of cysts.

PKB (Protein Kinase B). This is a member of a novel subgroup of serine/threonine kinases. *Giardia* PKB possesses a transmembrane domain in the catalytic region and a probable nuclear localization signal in the regulatory domain. It is constitutively expressed and is up-regulated during encystation [42].

PKC (Protein Kinase C): Several isoforms of this enzyme have been identified in *Giardia*, which show changes in expression during encystation. Selective PKC inhibitors blocked the encystation in a dose-dependent manner [43].

More deep studies are necessary to link these elements to each other, in order to improve the knowledge of signal transduction in *G. lamblia*.

ADI (Arginine Deiminase): This enzyme is part of the energy metabolism of the parasite but recently was reported that play multiple roles in signal transduction pathways leading to the control of encystation and antigenic variation due to the potential of this enzyme to citrullinate arginines present in *Giardia* proteins [44]. However, a recent report casts doubt about this additional function of *Giardia* ADI since recombinant dimeric enzymes were unable to perform this process reported to be caused by protein arginine deiminases (PADs) in higher organisms [45].

It is clear that from most of the cellular processes studied during adaptation and differentiation of this parasite, signal transduction pathways are among the least understood and, therefore, further studies are highly necessary to decipher the mechanisms of sensing the stimulus of encystation and excystation, the regulation of developmentally regulated expression of certain genes, the mechanisms controlling the cell cycle, and, if any, the control of antigenic variation.

3.3. Protein trafficking during encystation

The first structural manifestation of encystation is the formation of the encystation-specific secretory vesicles (ESV) in the cytoplasm of encysting trophozoites [11]. ESVs are typical secretory granules, with dense content, surrounded by a membrane [2]. This event is an example of regulated transport of cyst wall components. Nevertheless, secretory granules in higher eukaryotes form in the *trans* face of the Golgi apparatus [46]. Thus, how *Giardia* forms these granules without a Golgi apparatus is an intriguing question. Two major mechanisms have been proposed for trafficking throughout the Golgi complex in higher eukaryotes: vesicular transport between cisternae and cisternal maturation [47]. Hehl's group hypothesized that the ESVs are the Golgi equivalent in *Giardia* based on several observations [48]. However, since protein sorting and packaging also occur in non-encysting trophozoites, we prefer the idea that the absence of the typical Golgi apparatus in *Giardia* is due to the lack of glycosyl transferases, which are known to be present in different cisternae to perform a sequential addition of carbohydrates to complex glycoproteins [10].

Regarding the biogenesis of the ESVs, CWPs form disulfide-bonded heterodimers and oligomers soon after their synthesis; a key distinction between them is the presence of

a C-terminal 121-amino acid basic extension in CWP2 that is present when CWP2 is within the ESVs but is absent when the protein is in the cyst wall [13]. It has been shown that the tail in CWP2 is necessary for ESV formation, which is then processed by a cysteine protease later during encystation [10].

An extra component of the cyst wall has been described recently, a High-Cysteine Non-VSP Cyst protein, HCNCp [49]. This protein found in vegetative trophozoites, is up-regulated during encystation and colocalizes with ESVs in encysting trophozoites and with the cyst wall of mature cysts. The function of HCNCp during encystation is unclear. In the genome of the parasite, there are dozens of homolog genes that resemble VSPs (see later) but differ in their cytoplasmic tails [49]. From those, only this protein seems to be induced during encystation. Nothing is known about the other members of this family.

Another relevant issue is how *Giardia* transports the components of the cyst wall avoiding their intracellular polymerization [2]. A *Giardia* Granule-Specific-Protein (GSP) has been identified in the lumen of ESVs capable of binding calcium ions. GSP may be involved in calcium sensing to prevent the premature assembly of CWPs within ESVs and regulating the exocytic process [50].

The study of the cyst wall assembly is very difficult, probably due to its rapid and synchronous nature [2]. Nothing is known about the actual stimulus that triggers granule release and the molecular basis of this process is poorly understood.

The first morphological manifestation of this process *in vitro* is the detachment of the trophozoites and the change from pear-shaped to round or oval. Apparently, cytoskeletal structures like the ventral disk and the flagella are not necessary in the process, but are essential in the opposite process, excystation [1]. Early during encystation, the flagella are gradually internalized and ventral disk partially disrupted and maintained as fragments in the cytoplasm of the cell. Subsequently, over the dorsal and ventral surface, irregularly shaped bundles of variable extension begin to form, which are composed of fibrillar materials that gradually increase in size. These structures form multiples areas of coalescence that overlap progressively and turn the cell into a rounded form, completely covered with filamentous meshwork of about 300 nm [2].

Unfortunately, how the cyst wall is assembled remains unknown. Do all the ESVs fuse with the plasma membrane simultaneously? How is the fibrillar structure of the cyst wall deposited at the plasma membrane and not lost in the environment? How do the carbohydrate portions of the cyst wall interact with the CWPs? These are some of the questions that remain unsolved regarding this interesting process in which the machinery responsible for the production of the cyst wall is generated intracellularly, but the deposition and maturation of the wall occurs outside the cell. Further studies linking the known composition of the cyst wall and the morphological observations regarding its building are necessary.

4. The molecular mechanism of *Giardia* excystation

The infection of *Giardia* begins when cysts enter in a susceptible host [1,2]. Excystation is the process that consists of the release of trophozoites from the cysts, which proliferate in the small intestine. Some authors define to the recently excysted trophozoite as excyzoites, which have an oval shape and four nuclei that quickly divide (within 15–30 min) and subsequently undergo complete cell division (cytokinesis and karyokinesis) to give four binucleated trophozoites [21].

In the host, excystation starts in the stomach, when the cyst detects a high concentration of H^+, and is completed in the first part of the small intestine likely facilitated by pancreatic proteases and the slightly alkaline pH of the duodenum [1]. This is a rapid and coordinated event since, if excystation occurs prematurely in the stomach, the low pH would destroy the nascent trophozoites. On the other hand, if it occurs in the last part of the small intestine or in the colon, the low concentration of lipid and other nutrients would prevent the establishment of the infection [9].

There is no time for transcription or translation of new proteins during the passage of the cysts through the stomach; for this reason, rapid cell signaling in response to the excystation stimulus and the existence of preformed cytoskeleton structures are essential to establish the infection in a new host [8]. In this regard, protein kinase A activity and calcium signaling pathways have been reported to play a role in this process [51,52]. In addition, the cytoskeleton of *Giardia* is a key player in the infection process and its assembly is finely coordinated during encystation–excystation passages [7]. The four pairs of flagella are intact within the cyst, which is essential for the trophozoites during excystation because the movement of the flagella coincides with the release of the trophozoite from the cyst [1]. Another key component is the ventral disk; this structure is disassembled during encystation and stored as fragments in the cyst, which are quickly reassembled in a functional structure during excystation [9].

5. Molecular mechanisms involved in antigenic variation

Bacterial, protozoan and fungal pathogens from distant evolutionary lineages have developed surprisingly similar mechanisms to avoid recognition by the host immune system and therefore maintain chronic and/or persistent infections [53]. Antigenic variation is one such mechanism, which is characterized by the continuous switching of surface antigenic molecules to evade the immune response generated by the host [6]. However, some free-living organisms, like *Paramecium* and *Tetrahymena*, rely on antigenic variation as well [54].

Giardia also undergoes antigenic variation. This process was described initially as an *in vitro* phenomenon but subsequently was demonstrated in human and laboratory animal infections [6]. *Giardia* trophozoites are entirely covered by a family of proteins known as Variant-specific Surface Proteins (VSPs) [6]. Only one VSP, from a repertoire of about 190 VSPs genes present in the parasite genome, is expressed on the surface at a particular time [55], except during the switching process [6] and during encystation [56]. Switching of one VSP for another occurs spontaneously every 6–13 generations, even in the absence of immunological pressure [6].

VSPs are cysteine-rich proteins containing many CXX motifs, one or two GGCY motifs, a Zn-finger motif, a conserved transmembrane domain and an invariant cytoplasmic tail containing only five amino acids, CRGKA [6]. The amino terminal portion is variable and constitutes the interface between *Giardia* and the host [6]. VSPs are resistant to intestinal proteases, so one additional function of these proteins could be promoting the survival of the trophozoite in the protease-rich environment of the small intestine [6].

5.1. Gene regulation of antigenic variation

Two general types of mechanisms that control antigenic variation have been described in microbial systems: genetic and epigenetic mechanisms [53].

During the last years, several laboratories have been extensively studying the mechanism controlling antigenic variation in *G. lamblia*. Neither DNA sequence alterations nor DNA rearrangements have been described for VSP expression. VSP genes have no introns, their 5′ and 3′ UTRs are extremely short, presenting no evident homology and no promoter sequences have been described for this family of genes. A recent report demonstrated that expression of the VSP is independent of the 5′ and 3′ flanking sequences, postulating that an epigenetic mechanism is involved in antigenic variation of *Giardia* [57].

A more recent report postulated that a post-transcriptional gene silencing (PTGS) mechanism similar to RNA interference (RNAi) regulates VSP expression. Using RNA isolated from a particular clone that expresses a unique VSP, the simultaneous transcription of several VPS genes, although only one VSP mRNA corresponding to the VSP that is expressed at the trophozoite's plasma membrane, accumulates in the cytoplasm. Antisense VSP transcripts complementary to the sense strand of VSP were identified that were produced in the cytoplasm by the activity of an RNA-dependent RNA-polymerase (RdRP). Sense and antisense RNA form double-stranded RNA complexes (dsRNAs), which are the target for the action of the dsRNA endonuclease Dicer. It was shown that this enzyme generates small RNAs of 21–25-nt in length from transcribed but not translated VSP mRNAs. An Argonaute gene (AGO), a member of RNAi, is present in *Giardia* and it is possible that this molecule is part of the RISC complex that collaborates with the silencing process of VSP by Dicer. Importantly, when these key components of the RNAi machinery, Dicer and RdRP, were knocked-down, trophozoites expressing more than one particular VSP at the same time on their surface were generated [55].

How *Giardia* selects one or another VSP or how changes the expression of VSPs are the next question to study. Perhaps, an unbalance of the RNAi pathways due to a different VSP transcript concentration, the presence of additional molecules, changes in the chromatin state, or other epigenetic mechanisms may be acting in this fascinating process [55].

Recently, Saraiya and Wang [58] described the first snoRNA capable of producing miRNAs in any eukaryote. The miRNA targeted many different *Giardia* genes, including 22 VSPs genes, suggesting that this mechanism may be involved in controlling antigenic variation. These authors suggested that other still undiscovered miRNAs might be regulating many cellular processes in *Giardia*, including antigenic variation. Undoubtedly, the small RNAs might contribute to the fine-tuning regarding what VSP from the repertoire might be selected for expression in individual trophozoites [55].

6. Conclusion and perspectives

Recently, important advances in our knowledge of the biology of *Giardia* have been reported. Generation of the full sequence of the *Giardia* genome of the WB isolate (assemblage A) [3] has produced novel and exciting findings regarding the molecular mechanisms of protein trafficking and antigenic variation, as well as new ways to understand the relationship of this organism with the host. More recently, the draft genome sequencing of the other assemblage that infect humans (assemblage B) has been reported and suggested that the GS isolate, which belongs to this group, may represent a different species [59]. This finding will provide new evidence about the differences in the biology, infectivity and host specificity of *Giardia*. In fact, this organism is full of surprises and many important issues remain poorly understood or controversial. Since *Giardia* occupies a unique phylogenetic position, answers to these questions will provide information about the evolution of higher eukaryotes, but, since *Giardia* is a obligate parasite, a better understanding of the structure/composition relationship of key molecular processes and organelles may provide new clues of what essential components are needed when an organism colonizes a host and which are dispensable. The use of *Giardia* as a model system undoubtedly will generate information to understand the biology of other pathogenic organisms and the host response to them.

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References

[1] R.D. Adam, Biology of *Giardia lamblia*. Clin. Microbiol. Rev. 14 (2001) 447–475.
[2] H.D. Lujan, M.R. Mowatt, T.E. Nash, Mechanisms of *Giardia lamblia* differentiation into cysts. Microbiol. Mol. Biol. Rev. 61 (1997) 294–304.
[3] H.G. Morrison, A.G. McArthur, F.D. Gillin, S.B. Aley, R.D. Adam, G.J. Olsen, A.A. Best, W.Z. Cande, F. Chen, M.J. Cipriano, B.J. Davids, S.C. Dawson, H.G. Elmendorf, A.B. Hehl, M.E. Holder, S.M. Huse, U.U. Kim, E. Lasek-Nesselquist, G. Manning, A. Nigam, J.E. Nixon, D. Palm, N.E. Passamaneck, A. Prabhu, C.I. Reich, D.S. Reiner, J. Samuelson, S. G. Svard, M.L. Sogin, Genomic minimalism in the early diverging intestinal parasite *Giardia lamblia*. Science 317 (2007) 1921–1926.

[4] H.G. Morrison, A.J. Roger, T.G. Nystul, F.D. Gillin, M.L. Sogin, *Giardia lamblia* expresses a proteobacterial-like DnaK homolog. Mol. Biol. Evol. 18 (2001) 530–541.
[5] J. Samuelson, S. Banerjee, P. Magnelli, J. Cui, D.J. Kelleher, R. Gilmore, P.W. Robbins, The diversity of dolichol-linked precursors to Asn-linked glycans likely results from secondary loss of sets of glycosyltransferases. Proc. Natl. Acad. Sci. U S A 102 (2005) 1548–1553.
[6] T.E. Nash, Surface antigenic variation in *Giardia lamblia*. Mol. Microbiol. 45 (2002) 585–590.
[7] A.J. Solari, M.I. Rahn, A. Saura, H.D. Lujan, A unique mechanism of nuclear division in *Giardia lamblia* involves components of the ventral disk and the nuclear envelope. Biocell 27 (2003) 329–346.
[8] H.G. Elmendorf, S.C. Dawson, J.M. McCaffery, The cytoskeleton of *Giardia lamblia*. Int. J. Parasitol. 33 (2003) 3–28.
[9] D. Palm, M. Weiland, A.G. McArthur, J. Winiecka-Krusnell, M.J. Cipriano, S.R. Birkeland, S.E. Pacocha, B. Davids, F. Gillin, E. Linder, S. Svard, Developmental changes in the adhesive disk during *Giardia* differentiation. Mol. Biochem. Parasitol. 141 (2005) 199–207.
[10] N. Gottig, E.V. Elias, R. Quiroga, M.J. Nores, A.J. Solari, M.C. Touz, H. D. Lujan, Active and passive mechanisms drive secretory granule biogenesis during differentiation of the intestinal parasite *Giardia lamblia*. J. Biol. Chem. 281 (2006) 18156–18166.
[11] H.D. Lujan, M.C. Touz, Protein trafficking in *Giardia lamblia*. Cell. Microbiol. 5 (2003) 427–434.
[12] J. Tovar, G. Leon-Avila, L.B. Sanchez, R. Sutak, J. Tachezy, M. van der Giezen, M. Hernandez, M. Muller, J.M. Lucocq, Mitochondrial remnant organelles of *Giardia* function in iron–sulphur protein maturation. Nature 426 (2003) 172–176.
[13] H.D. Lujan, M.R. Mowatt, J.T. Conrad, B. Bowers, T.E. Nash, Identification of a novel *Giardia lamblia* cyst wall protein with leucine-rich repeats. Implications for secretory granule formation and protein assembly into the cyst wall. J. Biol. Chem. 270 (1995) 29307–29313.
[14] C.H. Sun, J.M. McCaffery, D.S. Reiner, F.D. Gillin, Mining the *Giardia lamblia* genome for new cyst wall proteins. J. Biol. Chem. 278 (2003) 21701–21708.
[15] S. Ghosh, M. Frisardi, R. Rogers, J. Samuelson, How *Giardia* swim and divide. Infect. Immun. 69 (2001) 7866–7872.
[16] M. Benchimol, Participation of the adhesive disc during karyokinesis in *Giardia lamblia*. Biol. Cell 96 (2004) 291–301.
[17] M.S. Sagolla, S.C. Dawson, J.J. Mancuso, W.Z. Cande, Three-dimensional analysis of mitosis and cytokinesis in the binucleate parasite *Giardia intestinalis*. J. Cell. Sci. 119 (2006) 4889–4900.
[18] B. Piva, M. Benchimol, The median body of *Giardia lamblia*: an ultrastructural study. Biol. Cell 96 (2004) 735–746.
[19] K.S. Kabnick, D.A. Peattie, In situ analyses reveal that the two nuclei of *Giardia lamblia* are equivalent. J. Cell. Sci. 95 (Pt 3) (1990) 353–360.
[20] L.Z. Yu, C.W. Birky Jr., R.D. Adam, The two nuclei of *Giardia* each have complete copies of the genome and are partitioned equationally at cytokinesis. Eukaryot. Cell 1 (2002) 191–199.
[21] R. Bernander, J.E. Palm, S.G. Svard, Genome ploidy in different stages of the *Giardia lamblia* life cycle. Cell. Microbiol. 3 (2001) 55–62.
[22] S.C. Dawson, M.S. Sagolla, W.Z. Cande, The cenH3 histone variant defines centromeres in *Giardia intestinalis*. Chromosoma 116 (2007) 175–184.
[23] M.K. Poxleitner, M.L. Carpenter, J.J. Mancuso, C.J. Wang, S.C. Dawson, W.Z. Cande, Evidence for karyogamy and exchange of genetic material in the binucleate intestinal parasite *Giardia intestinalis*. Science 319 (2008) 1530–1533.
[24] M. Benchimol, *Giardia lamblia*: behavior of the nuclear envelope. Parasitol. Res. 94 (2004) 254–264.
[25] J. Guo, Y. Chen, K. Zhou, J. Li, Distribution of rDNA in the nucleus of *Giardia lamblia*: detection by Ag-I silver stain. Biotech. Histochem. 80 (2005) 31–34.
[26] L.F. Jimenez-Garcia, G. Zavala, B. Chavez-Munguia, P. Ramos-Godinez Mdel, G. Lopez-Velazquez, L. Segura-Valdez Mde, C. Montanez, A.B. Hehl, R. Arguello-Garcia, G. Ortega-Pierres, Identification of nucleoli in the early branching protist *Giardia duodenalis*. Int. J. Parasitol. 38 (2008) 1297–1304.
[27] T. Lauwaet, B.J. Davids, D.S. Reiner, F.D. Gillin, Encystation of *Giardia lamblia*: a model for other parasites. Curr. Opin. Microbiol. 10 (2007) 554–559.
[28] F.D. Gillin, D.S. Reiner, M.J. Gault, H. Douglas, S. Das, A. Wunderlich, J.F. Sauch, Encystation and expression of cyst antigens by *Giardia lamblia in vitro*. Science 235 (1987) 1040–1043.
[29] H.D. Lujan, M.R. Mowatt, L.G. Byrd, T.E. Nash, Cholesterol starvation induces differentiation of the intestinal parasite *Giardia lamblia*. Proc. Natl. Acad. Sci. U S A 93 (1996) 7628–7633.
[30] M.S. Brown, J.L. Goldstein, The SREBP pathway: regulation of cholesterol metabolism by proteolysis of a membrane-bound transcription factor. Cell 89 (1997) 331–340.
[31] T.S. Worgall, S.R. Davis-Hayman, M.M. Magana, P.M. Oelkers, F. Zapata, R.A. Juliano, T.F. Osborne, T.E. Nash, R.J. Deckelbaum, Sterol and fatty acid regulatory pathways in a *Giardia lamblia*-derived promoter: evidence for SREBP as an ancient transcription factor. J. Lipid. Res. 45 (2004) 981–988.
[32] A.A. Best, H.G. Morrison, A.G. McArthur, M.L. Sogin, G.J. Olsen, Evolution of eukaryotic transcription: insights from the genome of *Giardia lamblia*. Genome Res. 14 (2004) 1537–1547.
[33] S.R. Davis-Hayman, J.R. Hayman, T.E. Nash, Encystation-specific regulation of the cyst wall protein 2 gene in *Giardia lamblia* by multiple cis-acting elements. Int. J. Parasitol. 33 (2003) 1005–1012.
[34] C.H. Sun, D. Palm, A.G. McArthur, S.G. Svard, F.D. Gillin, A novel Myb-related protein involved in transcriptional activation of encystation genes in *Giardia lamblia*. Mol. Microbiol. 46 (2002) 971–984.
[35] C.H. Wang, L.H. Su, C.H. Sun, A novel ARID/Bright-like protein involved in transcriptional activation of cyst wall protein 1 gene in *Giardia lamblia*. J. Biol. Chem. 282 (2007) 8905–8914.
[36] C.H. Sun, L.H. Su, F.D. Gillin, Novel plant-GARP-like transcription factors in *Giardia lamblia*. Mol. Biochem. Parasitol. 146 (2006) 45–57.
[37] Y.J. Pan, C.C. Cho, Y.Y. Kao, C.H. Sun, A Novel WRKY-like protein involved in transcriptional activation of cyst wall protein genes in *Giardia lamblia*. J. Biol. Chem. 284 (2009) 17975–17988.
[38] L.H. Su, G.A. Lee, Y.C. Huang, Y.H. Chen, C.H. Sun, Neomycin and puromycin affect gene expression in *Giardia lamblia* stable transfection. Mol. Biochem. Parasitol. 156 (2007) 124–135.
[39] H.G. Elmendorf, S.M. Singer, T.E. Nash, The abundance of sterile transcripts in *Giardia lamblia*. Nucleic Acids Res. 29 (2001) 4674–4683.
[40] J.G.T. Ellis, M. Davila, R. Chakrabarti, Potential involvement of extracellular signal-regulated kinase 1 and 2 in encystation of a primitive eukaryote, *Giardia lamblia*. Stage-specific activation and intracellular localization. J. Biol. Chem. 278 (2003) 1936–1945.
[41] T. Lauwaet, B.J. Davids, A. Torres-Escobar, S.R. Birkeland, M.J. Cipriano, S.P. Preheim, D. Palm, S.G. Svard, A.G. McArthur, F.D. Gillin, Protein phosphatase 2A plays a crucial role in *Giardia lamblia* differentiation. Mol. Biochem. Parasitol. 152 (2007) 80–89.
[42] K.T. Kim, M.T. Mok, M.R. Edwards, Protein kinase B from *Giardia intestinalis*. Biochem. Biophys. Res. Commun. 334 (2005) 333–341.
[43] M.L. Bazan-Tejeda, R. Arguello-Garcia, R.M. Bermudez-Cruz, M. Robles-Flores, G. Ortega-Pierres, Protein kinase C isoforms from *Giardia duodenalis*: identification and functional characterization of a beta-like molecule during encystment. Arch. Microbiol. 187 (2007) 55–66.
[44] M.C. Touz, A.S. Ropolo, M.R. Rivero, C.V. Vranych, J.T. Conrad, S.G. Svard, T.E. Nash, Arginine deiminase has multiple regulatory roles in the biology of *Giardia lamblia*. J. Cell. Sci. 121 (2008) 2930–2938.
[45] Z. Li, L. Kulakova, L. Li, A. Galkin, Z. Zhao, T.E. Nash, P.S. Mariano, O. Herzberg, D. Dunaway-Mariano, Mechanisms of catalysis and inhibition operative in the arginine deiminase from the human pathogen *Giardia lamblia*. Bioorg. Chem. 37 (2009) 149–161.
[46] B. Borgonovo, J. Ouwendijk, M. Solimena, Biogenesis of secretory granules. Curr. Opin. Cell. Biol. 18 (2006) 365–370.
[47] K. Matsuura-Tokita, M. Takeuchi, A. Ichihara, K. Mikuriya, A. Nakano, Live imaging of yeast Golgi cisternal maturation. Nature 441 (2006) 1007–1010.
[48] M. Marti, A.B. Hehl, Encystation-specific vesicles in *Giardia*: a primordial Golgi or just another secretory compartment? Trends Parasitol. 19 (2003) 440–446.

[49] B.J. Davids, D.S. Reiner, S.R. Birkeland, S.P. Preheim, M.J. Cipriano, A. G. McArthur, F.D. Gillin, A new family of giardial cysteine-rich non-VSP protein genes and a novel cyst protein. PLoS One 1 (2006) e44.

[50] M.C. Touz, N. Gottig, T.E. Nash, H.D. Lujan, Identification and characterization of a novel secretory granule calcium-binding protein from the early branching eukaryote *Giardia lamblia*. J. Biol. Chem. 277 (2002) 50557−50563.

[51] E.S. Abel, B.J. Davids, L.D. Robles, C.E. Loflin, F.D. Gillin, R. Chakrabarti, Possible roles of protein kinase A in cell motility and excystation of the early diverging eukaryote *Giardia lamblia*. J. Biol. Chem. 276 (2001) 10320−10329.

[52] D.S. Reiner, M.L. Hetsko, J.G. Meszaros, C.H. Sun, H.G. Morrison, L.L. Brunton, F.D. Gillin, Calcium signaling in excystation of the early diverging eukaryote, *Giardia lamblia*. J. Biol. Chem. 278 (2003) 2533−2540.

[53] K.W. Deitsch, S.A. Lukehart, J.R. Stringer, Common strategies for antigenic variation by bacterial, fungal and protozoan pathogens. Nat. Rev. Microbiol. 7 (2009) 493−503.

[54] M.C. Simon, H.J. Schmidt, Antigenic variation in ciliates: antigen structure, function, expression. J. Eukaryot. Microbiol. 54 (2007) 1−7.

[55] C.G. Prucca, I. Slavin, R. Quiroga, E.V. Elias, F.D. Rivero, A. Saura, P. G. Carranza, H.D. Lujan, Antigenic variation in *Giardia lamblia* is regulated by RNA interference. Nature 456 (2008) 750−754.

[56] P.G. Carranza, G. Feltes, A. Ropolo, S.M. Quintana, M.C. Touz, H.D. Lujan, Simultaneous expression of different variant-specific surface proteins in single *Giardia lamblia* trophozoites during encystation. Infect. Immun. 70 (2002) 5265−5268.

[57] L. Kulakova, S.M. Singer, J. Conrad, T.E. Nash, Epigenetic mechanisms are involved in the control of *Giardia lamblia* antigenic variation. Mol. Microbiol. 61 (2006) 1533−1542.

[58] A.A. Saraiya, C.C. Wang, snoRNA, a novel precursor of microRNA in *Giardia lamblia*. PLoS Pathog 4 (2008) e1000224.

[59] O. Franzén, J. Jerlström-Hultqvist, E. Castro, E. Sherwood, J. Ankarklev, D. S. Reiner, D. Palm, J.O. Andersson, B. Andersson, S.G. Svärd, Draft genome sequencing of *Giardia intestinalis* assemblage B isolate GS: is human giardiasis caused by two different species? PLoS Pathog. 5 (2009) e1000560.