

Encystation of *Giardia intestinalis*—a Journey from the Duodenum to the Colon

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Abstract The intestinal protozoan parasite *Giardia intestinalis* has a simple life cycle consisting of disease-causing trophozoites and infectious cysts. The parasite differentiates into the cyst stage (encystation) when duodenal trophozoites are swept further down in the small intestine. The encystation process can be divided into an early and a late phase. Expressions of cyst wall sugars and proteins are induced early during encystation, and *Giardia* has developed special transport systems for cyst wall material. This is combined with disassembly of the flagella and adhesive disc. In the later phase of encystation, nuclei are divided, DNA is replicated, and the parasites prepare for the next step in differentiation (excystation) via large changes in gene expression. Even if many aspects of the encystation process have been studied and much information has been gained during the last years, much still remains to be explored regarding this highly complex process.

Keywords Protozoa · Differentiation · Encystation-specific vesicle · Cyst · Cyst wall protein

Introduction

The intestinal protozoan parasite *Giardia intestinalis* (synonyms *Giardia lamblia* and *Giardia duodenalis*) is distributed

worldwide and estimated to cause 280 million symptomatic infections (giardiasis) annually [1]. The parasite is spread most often via contaminated water, and many developing countries are considered endemic regions. Symptoms of infection are variable but typically include watery diarrhea, nausea, gastric pain, and weight loss [2]. The first signs of infection appear after 6–15 days, and giardiasis is usually treated with metronidazole or other nitroimidazoles [3]. The parasite is noninvasive and secretes no known toxins, and there is currently limited understanding about the disease-causing mechanisms [2].

The parasite has a simple life cycle with two main stages, the trophozoite and the cyst, and two intermediary stages, encyzoites and excyzoites [4] (Fig. 1). The infection starts by host ingestion of infectious cysts, which are triggered to excyst by the acidic milieu in the stomach and the presence of bile and trypsin in the duodenum. The emerging excyzoites undergo cytokinesis twice, without an intervening DNA replication, giving rise to four trophozoites [5]. The binucleated trophozoites attach to the intestinal epithelium, using an adhesive disc, where they proliferate and cause disease. The parasite uses antigenic variation by expressing different variant surface-specific proteins (VSPs) to escape adaptive immunity and to prolong infection [4]. Encystation starts when the parasite is swept away further down in the small intestine, and the process can be divided into an early and late phase. The early phase involves the formation of encystation-specific vesicles (ESVs) that exclusively transport cyst wall proteins (CWPs) to the membrane for building of the cyst wall. The ESV containing cell (encyzoite) starts to change shape, and the adhesive disc together with the flagella is internalized [6]. In the late phase of encystation, the nuclei divide and undergo a round of DNA replication, giving the mature cyst four tetraploid nuclei [5]. The last step of encystation is a maturation step where the cyst wall filaments are cross-linked to generate a compact,

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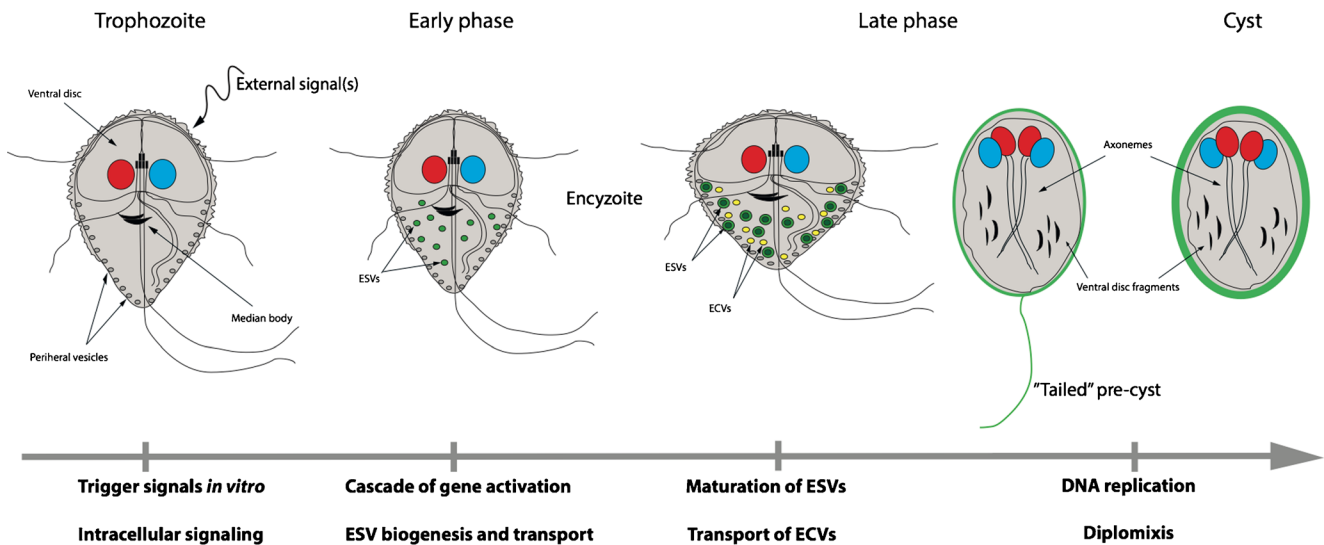


Fig. 1 Summarizing image showing the gradual transformation from the motile trophozoite via encyzoite to the final cyst stage. The trophozoite senses the external environment, and encystation is induced via not fully understood intracellular pathways. The trophozoite passes a point in early differentiation after which it is no longer possible to revert back to the proliferating stage. Transcription of encystation-specific genes is activated by different transcription factors (e.g., Myb2), among them, the three cyst wall proteins (CWP1–3). The transcription appears to be controlled on multiple levels, e.g., by epigenetic mechanisms. The production of CWPs is dramatically increased, and the transportation in encystation vesicles (ESVs) begins in early phase of the process. These vesicles undergo maturation steps after leaving the ER during which proteolytic processing of CWP2 leads to the formation of a condensed core. The

other component of the cyst wall, the UDP-N-acetylgalactoseamine (UDP-GalNac) sugar, is also synthesized and secreted via encystation-positive carbohydrate vesicles (ECVs). The mechanism behind assembly of the cyst wall remains unknown. Late during encystation, the cell rounds up as it approaches dormancy and the ventral disc together with the flagella is disassembled. Often, pre-cyst stages with a “tail” can be observed in encystation. Two rounds of DNA replication occur without cytokinesis rendering a cyst with four nuclei each with the genome ploidy of 4N. Interconnections between the nuclei in the cysts are formed, and genetic material can be exchanged through the process “diplomixis.” During excystation, each cell receives one pair of nonsister nuclei (as indicated by red and blue)

mature cell wall [6]. Cysts secreted in fecal material are effectively protected from environmental stress like UV [7] by the cyst wall and can survive several weeks in fresh water.

The encystation process is essential for transmission and survival of the parasite *G. intestinalis*. This review gives an update on recent findings regarding this fascinating cellular transformation and highlights areas where future research is needed.

Induction of Encystation

It is possible to study the entire life cycle of this parasite in vitro, and several encystation protocols have been developed for this purpose. The most used is the two-step protocol [8] where trophozoites are starved for bile prior to addition of encystation medium that contains high levels of porcine bile and lactic acid. Other protocols have also been developed which use cholesterol-depleted serum [9] or high levels of bovine bile [10], and these have been proven to be very effective in producing mature cysts. The protocols share the features of lipid starvation and an elevated pH, which are likely stimuli of the parasite encounter when relocated to the lower part of the small intestine in the host. It is not sufficient to only increase the pH of the growth media to induce encystation

[11•], so a combination of these two signals is required for encystation in vitro.

There is a very limited knowledge about how intracellular signaling is induced and mediated during encystation. *Giardia* possesses two homologs of extracellular regulated kinases 1 and 2 (ERK1 and ERK2), well-known MAP kinases involved in intracellular signaling in higher eukaryotes [12]. In *Giardia*, these proteins localize to different structures; ERK2 localizes to the nuclei and caudal flagella in trophozoites and appears mostly cytoplasmic in encysting cells, whereas ERK1 seems to be associated with the median body and the outer rim of the disc [13]. However, neither ERK1 nor ERK2 translocates to the nuclei during encystation, and so far, no transcription factors have been shown to be regulated by the giardial ERK proteins. Putative homologs of MEK and MEKK kinases are present in the genome of *Giardia*, but if they play a role in activating ERK1/2 remains to be investigated [13]. Even though several studies exist on individual signaling proteins [13–17], much remains to be explored to elucidate the complete signaling pathways for this parasite. Another interesting aspect is how long the encystation-inducing signals need to be present before the trophozoites commit to differentiation. A recent publication shows that trophozoites exposed to encystation medium for 3–6 h continue to encyst even if they are returned to normal growth medium [18], and hence,

commitment is an early event in the process. Around the same time (5–8 h), ESV formation is prominent among trophozoites in the population [11•, 19, 20••], indicating that when cyst wall production has reached a certain stage, the cells will complete the encystation process. This suggests that the parasite reaches a “point of no return” early in encystation (3–6 h) after which it is impossible to revert back to proliferation and differentiation will continue until mature cysts are generated. It also means that very early during encystation, there is a possibility for trophozoites to abort the process, if the surrounding environment becomes advantageous for growth.

The response to start encystation is heterogeneous among *Giardia* cells in the population in vitro; some of the cells will never induce encystation, whereas some spontaneously undergo cyst formation even in absence of encystation medium. In vivo experiments in gerbils using isolate WB show that trophozoites and encysting cells are distributed evenly in the small intestine [21]. The majority of cysts was found in the colon, but also present in last sections of the small intestine [21]. This suggests that there are a variety of cell stages present in vivo as well, where some cells do not undergo encystation and not all encysting cells complete the process. Moreover, different clones of the isolate WB undergo the encystation process differently [10, 21]. Adding to the complexity of how encystation is triggered, it seems that assemblage A parasites proliferate and differentiate better in vitro than parasites belonging to other assemblages [4]. At the same time, these isolates grow and differentiate well in vivo [4]. Perhaps, this is due to that vital signals are missing during the in vitro conditions but present in vivo, and therefore, much could be learned regarding the signaling and what triggers the encystation process by studying parasites from different assemblages both in vitro and in vivo.

Transcriptional Response During Encystation

The promoter regions of *Giardia* are short and A/T-rich without any identifiable TATA-box to initiate transcription [22]. The homolog to the TATA-binding protein (TBP) found in the genome is extremely divergent, and only four of the 12 general transcription initiation factors have been reported to have giardial homologs [23]. A few giardial transcription factors have been studied in more detail, especially their role in encystation. Much focus has been put on the regulation of CWPs and enzymes involved in cyst wall biosynthesis. The first transcription factor identified and characterized is a Myb2-like protein. The level of giardial Myb2 (gMyb) increases during encystation, and it localizes to the nuclei [24]. gMyb recognizes and can bind the sequence C(T/A)AC(A/T)(G/C) which is found in the promoter regions of encystation-induced genes like *cwp1–3* and *g6pi-b*, an important enzyme in the synthesis of the $\beta(1-3)$ -*N*-acetyl-D-galactosamine

(GalNAc) sugars in the cyst wall, as well as for *gmyb2* itself [24]. Overexpression of gMyb2 leads to an increase of CWP1 on both the messenger RNA (mRNA) and protein level, and the capability to form mature cysts increases [25]. Deleting the Myb2-binding site of the promoter region of *g6pi-b* leads to a reduction in promoter activity during encystation for this gene [24]. Moreover, when the Myb2-binding site is mutated to reduce Myb2 binding, the promoter activity is reduced during encystation. Hence, this site is important for promoter activity and the positive autoregulation of this transcription factor [25]. Interestingly, there are indications that the CWP genes might be silenced in trophozoites by a repressor mechanism allowing encystation-specific expression. Only 64 nucleotides upstream of the start codon of CWP-2 are needed for encystation-specific expression of luciferase [26]. When deleting certain elements within this region (–64 to –23), the CWP2 promoter increased its expression in trophozoites, suggesting the presence of a negative *cis*-acting element [26]. The authors also reported that the region contained a positive *cis*-acting element, as deletion of another area (–23 to –10), led to less expression in encysting cells. Several other transcription factors have been investigated and reported to play a role in the regulation of the expression of cyst wall proteins [27–33]. It remains to be investigated if and how these interact to modulate the massive induction of these genes. It would also be very interesting to knockout the transcription factors to see how that affects the differentiation process. So far, knockout experiments have been lacking in this tetraploid parasite, and it would be a vital tool to investigate several aspects of the biology of *Giardia*. It is important for the parasite to have a tight regulation of the encystation-specific genes since it would otherwise affect replication in the upper small intestine. Most likely, this is performed at multiple levels and further studies will show how this is accomplished, e.g., via transcription factors combined with chromatin structure and small RNAs (see below).

To date, there are two studies that describe the overall transcriptional responses during encystation. In the first study, the transcriptome of the entire life cycle was investigated using SAGE technology [34•]. Only 42 transcripts were found to be upregulated during encystation in this study: the three CWPs, several enzymes involved in UDP-GalNAc synthesis, the excyzoite surface protein high cysteine nonvariant cyst protein (HCNCp), and 13 hypothetical genes [34•]. However, two different encystation protocols were combined in this study (trophozoites to 42-h encystation via the two-step method and cysts via the high bile method). In the other study, the transcriptional response was studied during the first 7 h of encystation during which the CWPs are synthesized and transported from the ER in ESVs for maturation [11•]. Microarrays were used, and to avoid any protocol-induced off target effects, the authors used two different encystation protocols. The two-step method induced 29 genes whereas the cholesterol-free serum method induced 37 genes early during

encystation, generating a core set of only 13 genes that are induced early during encystation [11•]. The core-upregulated genes all contain at least one Myb2-binding site, suggesting that the Myb-binding site is a signature motif for early phase encystation genes.

For additional information about temporal changes of gene expression during encystation and the regulation behind it, further studies are needed that include the early and later stages of the encystation process as well as mature cysts.

Changes of the Proteome During Encystation

The first overview of the *Giardia* proteome of encysting cells is available and gives an important complement to the existing knowledge of the transcriptional response [20••]. The study investigates the proteome during the first 14 h of encystation during which cells accumulate CWP in the ER and mature ESVs are formed. The *Giardia* proteome was reported to be overall robust during encystation with large number of overlapping proteins between time points. The largest differences were observed in the early phases of encystation, suggesting that the regulation on the protein level is strongest at this time [20••]. Comparing samples from 0 to 12 h shows similar distribution of proteins with predicted cellular localization for the mitosome, nucleus, and cytosolic proteins. There was a reduction for proteins predicted to traffic to or through the ER at 4 h, and the majority of secreted proteins found at the other time points were VSPs. An overall reduction of the VSP repertoire was found at 4 h, consistent with the decrease of ER-targeted proteins. The data indicate that the VSP diversity is affected by encystation [20••]. The high cysteine membrane proteins (HCMPs) are another group of predicted secreted proteins that also changed during encystation [20••]. Perhaps, these two groups of cysteine-rich surface proteins share a regulatory mechanism during this process. Proteins that significantly increased during encystation are involved in diverse functions such as protein folding, glycolysis, cytoskeleton regulatory components, and NEK kinases [20••]. Only modest changes were reported for the later time points (8–12 h) of differentiation, and it was reported that genes associated with carbohydrate biosynthesis were enriched at this time point. There were many hypothetical proteins reported in the data sets, reflecting the large number of genes present in parasite genomes with unknown function [20••].

Changes in the proteome involve new protein synthesis but also degradation. Proteasomes have been shown to interact with the ESV membrane early during encystation, suggesting a role for the proteasome in *Giardia* encystation [35]. Proteasomes with the regulatory 19S particle have been purified using a tandem affinity proteomic approach [36]. This revealed that *Giardia* proteasomes lack the Rpn13 Ubiquitin receptor, but the other receptor, Rpn10, was found. A recent

study showed localization of Rpn10 to the flagellar pores, nuclei, and cytosol in trophozoites. However, the association to the flagellar pores was not evident in encysting cells [37].

Ubiquitination and, in particular, the ubiquitin-activating enzyme E1 seem to be involved in the differentiation process. The expression of E1 is regulated on both transcriptional and protein level during encystation, and overexpression of the protein yielded increased encystation rate. The localization of the enzyme appears to be associated to the cyst wall and as cytoplasmic spots in mature cysts [38]. The SUMO modification is similar to ubiquitin, but not used for tagging proteins for degradation by the proteasome. SUMOylation of the metabolic enzyme arginine deiminase (ADI) improves translocation from the cytoplasm to the nuclei during late encystation. Inside the nuclei, ADI functions as a peptidyl arginine deiminase (PAD) and is suggested to downregulate the transcription of CWP2 [39]. The activity of acyltransferases and the reversible modification S-palmitoylation during encystation have been studied [40]. Several potential acyltransferases were found in the genome, and they are expressed in trophozoites as well as encysting parasites. During encystation, the levels of expression of CWPs and ESV formation differed among parasite lines overexpressing different acyltransferases, but proteomic analysis would be needed to find the targets of these acyltransferases and how these contribute during encystation [40].

To date, there are no proteomic studies that include the complete transformation from trophozoite to mature cyst. It will be important to expand the knowledge about changes in the proteome and connect it to the identified transcriptional changes during *Giardia* encystation. Large-scale analyses of post-translation modifications can complement this and generate a complete picture of encystation-induced gene expression changes.

Building of the Cyst Wall

The most striking change in secretion of proteins occurs during the early phase of encystation when massive amounts of cyst wall components are transported from the ER to the plasma membrane. The cyst wall material is transported in ESVs, and the formation and transport of these have been extensively studied [41, 42] since they were discovered [43]. The water-resistant insoluble cyst wall is made of 36 % protein and 63 % GalNAc homopolymer [44]. The cyst wall protein family consists of three members, which share several features [45, 46]. The presence of an N-terminal signal peptide targets them to the secretory pathway, and all three contain a central leucine-rich repeat (LRR) region important for sorting into the ESVs [45]. CWP1 and 2 carry five tandem LRRs whereas CWP3 has four complete and one incomplete repeat regions [46]. A cysteine-rich domain is found in the C-terminal region of the

CWPs, and CWP2 is different compared to the other two by the presence of a basic extension in this region [45]. CWPs peak in expression on the mRNA level at approximately 7-h post-induction, but an accumulation of newly synthesized protein appears in the ER after about 2-h post-induction. The cargo of ESVs is exclusively cyst wall material and no other secreted proteins, strongly suggest sorting in the ER prior to export [42]. *Giardia* lacks a constitutively expressed Golgi apparatus, and the features of ESVs indicate that these organelles act as a stage-induced *cis* Golgi. The trafficking process of cyst wall material, from the ER to the ESV, appears to be dependent on COPII coat formation and the small GTPase Sar1 [35]. Recently, it was shown that ER exit sites (ERES) induce ESV biogenesis since CWP1 depends on functional ERES for accurate trafficking [47••]. Furthermore, the colocalization of the nascent ESVs and ERES suggests a role of ERES in ESV biogenesis [47••]. The CWPs are not directly secreted after completion of ESV formation but, instead, delayed for several hours and converted into a trans-Golgi-like tubular vesicular network during late encystation [35]. This is presumably to allow post-translational modifications prior to secretion at the cell surface. A number of modifications have been reported such as formation of disulfide bonds, isopeptide-linkages, and addition of phospho-groups [48–50]. Another modification is the proteolytic processing of the basic extension of CWP2. Dual-tagged CWP2 and CWP/GFP chimeras have been used to study processing of the proteinaceous portion of the cyst wall material (CVM) in ESVs [51••]. The cell wall material (CWM) is partitioned into two distinct phases; CWP3 forms a condensed core together with the processed form of CWP2, and the mature N-terminal CWP2 together with CWP1 remains in fluid state until secretion. The fluid part is secreted rapidly whereas the condensed part of the CWM is secreted slowly for hours [51••]. This indicates that the formation of the cyst wall is a highly complex process.

A part from the CWPs, a few other proteins have been found to localize to the ESVs. Among them is an HCNCp that localized to ESVs and secreted to the plasma membrane-cyst wall interface and stayed on the surface of the excyzoite in the cyst [52]. Another group of cysteine-rich proteins (tenascin-like proteins) was also studied, and they appear to mainly localize not only to the cyst wall but also to the excyzoite surface [53].

The lysosomal-like peripheral vesicles (PVs) and ESVs were subjected to flow cytometry organelle sorting coupled to mass spectrometry aiming to find additional proteins associated to these organelles [54••]. The proteome data set from the ESV-enriched fraction revealed a candidate set of 72 proteins. Among these, many had ER localizations and the membranes of the two-organelle systems appear closely connected. Existing evidence of connections between ESVs allowing exchange of CWM makes it more challenging to define ESV

boundaries [35]. Several ribosomal proteins were enriched in the ESV fraction, and ribosomes were recruited to ESV membranes during differentiation. A possible explanation could be the presence of co-translational insertion of proteins from the ER across ESV membranes, but further experiments are needed for validation [54••].

While the CWPs are transported from the ER to the surface by ESVs, much less is known about the transport and incorporation of the UDP-GalNAc sugar in the cyst wall. This *Giardia*-unique carbohydrate is synthesized *de novo* by a pathway containing five enzymes of bacterial origin [55, 56] using endogenous glucose as starting molecule. The enzymes are transcriptionally and allosterically upregulated during encystation [55]. During the final step of synthesis, the UDP-GalNAc is polymerized by an activity named the “cyst wall synthetase” [57]. Even if evidence exists for the presence of this enzyme via activity measurements, it still remains unknown which gene(s) that encode this protein. The structure and assembly of the cyst wall have been under investigation, and the homopolymer of GalNAc appears as curled fibrils compressed by CWPs [58]. Both CWP1 and CWP2 can bind the fibrils, and the LRR of CWP1 is reported to have lectin-binding properties [58]. In the same study, the GalNAc homopolymer was visualized using the binding properties of recombinant CWP1 and reported to be present in small vesicles of encysting *Giardia*. These vesicles did not co-localize with ESVs, suggesting a different transportation pathway of the sugar components of the cyst wall [58]. In line with this result, a recent study proposes the presence of carbohydrate-positive vesicles (ECVs) in encysting parasites [59]. The authors found electron-translucent vesicles that stain positive for carbohydrate content, and binding of the lectin DBA indicated the presence of GalNAc [59]. Localization studies of putative the UDP-GlcNAc 4'-epimerase and a nucleotide sugar transporter were performed based on their presence in ESV proteomics data [54••]. The epimerase is associated to the ER and the transporter mainly to perinuclear ER and early ESVs pointing toward synthesis of UDP-GalNAc in the ER [54••].

Further studies are needed to clarify how the ESVs and ECVs are connected and to identify the mechanism behind cyst wall assembly.

Changes in Lipid Metabolism During Differentiation

The large changes in vesicle transport during encystation require production of new membrane structures. Being a parasite, *Giardia* has a limited range of metabolic pathways [56] and depends on uptake of lipids and fatty acids from the environment [60]. The sphingolipid ceramide cannot be synthesized *de novo* and is taken up by the parasite using clathrin-mediated pathways [61]. Only three synthesis-related genes

and two metabolic genes from the sphingolipid pathway are present in *Giardia* [62]. These genes are differentially expressed, and the glycosylceramide transferase (GlcT1) is only expressed in encysting parasites [62]. By modulating the levels of GlcT1, using a specific inhibitor, the levels of encystation and cyst production were reduced. This shows the importance of glucosylceramide synthesis during the process of encystation [62, 63]. Further studies using morpholino knockdown of the enzyme showed abolished ESV formation and generated nonviable cysts [64]. Most recently, the presence of cholesterol and GM ganglioside-enriched raft-like domains was shown in the plasma membrane of *Giardia* trophozoites [65•]. Lipid rafts are found in the plasma membrane of many eukaryotes and are involved in various processes e.g., cell adhesion, membrane signaling, and differentiation [66]. The cholesterol-binding drugs nystatin and filipin III disrupt the lipid rafts in *Giardia* trophozoites, interfere with ESV formation, and alter the morphology of cysts [65•]. It was suggested that the raft-like domains require cholesterol to maintain integrity, which appears critical for encystation [65•]. The exact function and potential involvement in signaling of the lipid rafts during encystation remain unknown.

Fusion of Nuclei Occurs During Late Encystation

The binucleated trophozoite alternates from a diploid (2x2N) to a tetraploid (2x4N) genome content during the cell cycle. The G2 phase is the longest phase of the cell cycle, and it contains the restriction point to start differentiating into the cyst form [5, 67]. During encystation, the two nuclei divide without cytokinesis forming a pre-cyst with ploidy of 4x2N followed by another round of replication to the final ploidy of 16N (4x4N) in the mature cyst [5]. During excystation, the excyzoite goes through cellular division twice without DNA replication, in the end, generating four trophozoites [5]. *Giardia* is considered to be asexual, but several homologs to genes involved with meiosis in other eukaryotes are found in the genome [4]. Moreover, the genome of WB isolate contains low levels of allelic sequence heterozygosity (ASH) which is surprising for an asexual organism that lack control of the buildup of differences between nuclei [56, 68]. This suggested that the nuclei can be connected and put the cyst nuclei into focus. Evidence for fusion of the nuclei and exchange of genetic material in the form of episomal plasmids was discovered during encystation [69]. The authors named this event “diplomixis,” and three meiosis genes were reported to be expressed late in encystation to facilitate homologous recombination [69]. Later, it was shown that chromosomally integrated markers were exchanged between nuclei during differentiation [70]. By using FISH to follow the integrated markers, it was shown that daughter trophozoites inherit one copy of each parental nucleus after excystation [70]. The

recombination events occurring during encystation by diplomixis could be involved in reducing ASH between nuclei [70].

The mechanisms behind the nuclear division were further investigated by [71]. Nuclear division occurs by semi-open mitosis, resulting in four daughter nuclei from two nonsister pairs. The pairs observed are interconnected with nuclear membrane bridges that remain in the excyzoites [71]. After division of excyzoite, each trophozoite inherits one pair of nonsister nuclei, in line with the model proposed by [70].

Regulation of Differentiation Is Likely to Occur at Multiple Levels

The fact that very few transcription factors have been found for this parasite (see above) might be an indication that the regulation of gene expression occurs at multiple levels. The process of encystation is complex and most likely entails sequential activation of genes required for successful development into the cyst stage. The details of this regulation remain incomplete.

Epigenetic changes are involved in the encystation process, and histone acetylation decreased during encystation [72]. The histone acetylation levels were modified using a histone deacetylase (HDAC) inhibitor resulting in increased levels of histone acetylation, alteration of gene expression, and blocked cyst formation [72]. The results clearly indicate that chromatin changes are part of dictating the transcriptional response of this parasite differentiation process. *Giardia* possesses several SF2 RNA helicases [73], and in higher eukaryotes, RNA helicases can interact with HDACs [74]. The expression level of two DEAD-box helicases increased greatly during encystation conditions. Authors also predict that helicases can be involved in the RNAi pathway present in *Giardia* [75] and participate in post-transcriptional silencing of genes [73].

Antigenic variation appears to be regulated by RNAi-related pathway and/or microRNAs [75, 76]. Recently, it was investigated if small RNAs can be involved in regulation of encystation [77•]. Two time points during encystation as well as trophozoites and cysts were analyzed by deep sequencing. Increasing levels of endogenous small interfering RNA (siRNA) were reported during the process originating from telomeric retrotransposons and from specific clusters in the genome [77•]. A knockdown strain of giardial Dicer was used in encystation and reported to be impaired in cyst formation, indicating a role of siRNAs in the process since Dicer is required for siRNA biogenesis. However, none of the targets predicted for the siRNAs has been experimentally verified. The siRNA data should be compared to transcriptional response of mRNA and protein expression to get a complete picture of their function during encystation. In addition, tRNA-derived siRNA was also found to increase during late

encystation, but their function during differentiation is difficult to speculate about without further studies [77•].

Conclusions

The complete life cycle of the parasite *G. intestinalis* can be studied in vitro [9, 10, 43] and has generated the possibility to study many vital aspects of encystation. However, the exact trigger and pathways responsible for induction of the process remain unknown. There are some indications of important players in the signaling pathway, but further studies using specific inhibitors are needed to investigate the exact role. Another possibility is to study the process in other assemblages, such as the GS isolate, or possibly even during in vivo conditions.

Many aspects of early encystation have been studied, including the biogenesis and transport of ESVs [41, 42]. It will be interesting to investigate the detail of how the carbohydrates are transported in ECVs [59] and the interactions between CWPs that must occur to build the cyst wall. Perhaps, the finding of the gene encoding the cyst wall synthetase will help to gain information regarding this process.

Transcriptional profiles coupled with proteomics ranging over the entire encystation process, including mature cysts, would improve current knowledge of temporal activation of genes and their protein product. Future studies are also needed to elucidate how genes are regulated during encystation, which appear to be orchestrated on multiple levels [72, 77•].

Lastly, the possibility to do knockout experiments on specific genes will improve the knowledge about the role certain proteins play during the journey in the small intestine from trophozoite to a cyst.

Compliance with Ethics Guidelines

Conflict of Interest Elin Einarsson and Staffan G. Svärd declare that they have no conflict of interest.

Human and Animal Rights and Informed Consent This article does not contain any studies with human or animal subjects performed by any of the authors.

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