Export of cyst wall material and Golgi organelle neogenesis in *Giardia lamblia* depends on ER exit sites

Carmen Faso¹, Christian Konrad¹⁵, Elisabeth M. Schraner² and Adrian B. Hehl¹*

¹Laboratory of Molecular Parasitology, Institute of Parasitology, University of Zurich, Winterthurerstrasse 266a, 8057 Zurich, Switzerland.
²Institutes of Veterinary Anatomy and Virology, University of Zurich, Winterthurerstrasse 266a, 8057 Zürich, Switzerland

*Current address: Indiana University School of Medicine, Departments of Biochemistry & Molecular Biology and Pharmacology & Toxicology, 635 Barnhill Drive, Indianapolis, IN, 46202, USA

Keywords: Golgi, Giardia, ER exit site, secretion, protein sorting, organelle evolution

Running title: ER exit sites in *Giardia lamblia*

*Corresponding author

Correspondence and requests for materials should be addressed to:

Adrian B. Hehl
Laboratory of Molecular Parasitology
Institute of Parasitology
University of Zurich
Winterthurerstrasse 266a
8057 Zürich, Switzerland

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/cmi.12054

© 2012 Blackwell Publishing Ltd
Abstract

*Giardia lamblia* parasitism accounts for the majority of cases of parasitic diarrheal disease, making this flagellated eukaryote the most successful intestinal parasite worldwide. This organism has undergone secondary reduction/elimination of entire organelle systems such as mitochondria and Golgi. However, trophozoite to cyst differentiation (encystation) requires neogenesis of Golgi-like secretory organelles named encystation specific vesicles (ESVs), which traffic, modify and partition cyst wall proteins (CWPs) produced exclusively during encystation.

In this work we ask whether neogenesis of Golgi-related ESVs during *G. lamblia* differentiation, similarly to Golgi biogenesis in more complex eukaryotes, requires the maintenance of distinct COPII-associated ER subdomains in the form of ER exit sites (ERES) and whether ERES are also present in non-differentiating trophozoites.

To address this question, we identified conserved COPII components in *G. lamblia* cells and determined their localization, quantity and dynamics at distinct ERES domains in vegetative and differentiating trophozoites. Analogous to ERES and Golgi biogenesis, these domains were closely associated to early stages of newly generated ESV. Ectopic expression of non-functional Sar1-GTPase variants caused ERES collapse and, consequently, ESV ablation, leading to impaired parasite differentiation.

Thus, our data show how ERES domains remain conserved in *G. lamblia* despite elimination of steady-state Golgi. Furthermore, the fundamental eukaryotic principle of ERES to Golgi/Golgi-like compartment correspondence holds true in differentiating Giardia presenting streamlined machinery for secretory organelle biogenesis and protein trafficking. However, in the Golgi-less trophozoites ERES exist as stable ER subdomains, likely as the sole sorting centers for secretory traffic.
Introduction

*Giardia lamblia* is a medically-relevant flagellated protozoan responsible for ca. 300 million cases of waterborne and occasionally food borne diarrheal disease every year (Yoshida *et al.*, 2011; Lane and Lloyd, 2002). Recently, this parasite has emerged as an attractive protozoan model system for the study of complex cellular processes due to its reduced and sequenced genome, simplified endomembrane system and gene expression machinery (Faso and Hehl, 2011; Morf *et al.*, 2010; Kolev and Ullu, 2009; Poxleitner *et al.*, 2008; Morrison *et al.*, 2007; Elmendorf *et al.*, 2003). Coupled to the ease with which the entire life cycle can be replicated in vitro, this Giardia provides us with a unique opportunity for the investigation of the minimal components required for secretory cargo transport, organellar formation and maintenance in a relatively uncluttered environment (Dacks and Field, 2007).

*G. lamblia* presents two life cycle stages, the cyst and the trophozoite. Following ingestion of cysts, trophozoites emerge and replicate in the small intestine in a process called excystation. Some trophozoites will differentiate by synthesizing and accumulating cyst wall material (CWM), detach from the intestinal lining and initiate encystation whereby flagellated individuals become environmentally-resistant cysts, thus completing the parasite’s life cycle (Faso and Hehl, 2011; Ankarklev *et al.*, 2010). Giardia differentiation from trophozoite to infectious cyst entails secretion of large amounts of extracellular biopolymer (the cyst wall) mainly consisting of 3 soluble secretory cyst wall proteins (CWPs 1-3) and a beta (1-3) N-acetylgalactosamine homopolymer (Hehl and Marti, 2004; Gerwig *et al.*, 2002). CWPs1-3 are synthesized in the ER and subsequently transferred to organelles termed encystation-specific vesicles (ESVs), arising at the onset of encystation. ESVs are considered Golgi cisterna analogues and have been shown to possess several Golgi-like features such as fragmentation following treatment with brefeldin A and physical association to coatamer components (Stefanic *et al.*, 2006; Marti *et al.*, 2003b). Within ESVs, which harbor exclusively cyst wall components as cargo, CWPs are modified and partitioned in two biophysically-distinct fractions within the organelles: a peripheral fluid phase surrounding a condensed core. In the later stages of encystation, the two fractions are secreted sequentially to the nascent cyst wall (Konrad *et al.*, 2010). Importantly, encystation and ESVs are unique in that, to date, they constitute the only example of a complex inducible secretory pathway with de novo synthesis of basic Golgi-like organelles for the sorting, maturation and deposition of a very specific set of secretory cargo molecules.
Secretory proteins in eukaryotic cells are generally trafficked from their site of synthesis at the rough endoplasmic reticulum (ER) to their destination in a complex quality-controlled process requiring at least two sorting steps, concentration and packaging into diverse membrane carriers and acquisition of complex post-translational modifications (Vazquez-Martinez et al., 2011; Schmidt and Stephens, 2010). In general, secreted proteins are co-translationally inserted into the ER membrane via the Sec61 translocon complex. Following quality control in the ER, proteins are then transported to the Golgi apparatus through which they transit, reaching the trans-Golgi network. Here, distinct secreted proteins are sorted to specific destinations, be it the extracellular space, the plasma membrane or subcellular compartments such as vacuoles and lysosomes.

Proteins which are exported from the ER mostly do so in COPII coated membrane carriers generated at specific ER domains termed ER exit sites, i.e. ERES or transitional ER (tER) in yeast) (Budnik and Stephens, 2009). The composition and function of the machineries which define these ER subdomains has been investigated in much detail over the past years. The biogenesis of a COPII transport intermediate begins with the recruitment of Sar1-GTPase to the cytosolic face of the ER membrane by its ER-bound GDP-GTP exchange factor (GEF) Sec12 (Bielli et al., 2005). Subsequently, Sec23/24 heterodimers associate to activated Sar1-GTPase forming a pre-budding complex, which is followed by aggregation of Sec13/31 heterodimers, causing membrane deformation and formation of a cargo-enriched coated vesicle. During these successive recruitment events, secretory cargo is contacted by Sec24 and enriched in the pre-budding complex (Bi et al., 2002). In a process which has not yet been mechanistically elucidated, the fully formed coat induces formation of a COPII vesicle containing secretory cargo surrounded by an inner and outer coat protein layer composed of Sec23/24 and Sec13/31 heterodimer, respectively (Lee and Miller, 2007). In addition to new cargo proteins, fusion of these ER-derived carriers provide membrane material to the cis-face of the Golgi and contribute to the biogenesis of the most proximal cisterna (Brandon and Sztul, 2004; Ward et al., 2001), although several studies indicate that other mechanisms such as fragment-driven biogenesis are also at play in the formation of the Golgi organelle (Pelletier et al., 2002; Shorter and Warren, 2002).

Our group has previously shown that ESV neogenesis and subsequent cyst wall formation is inhibited following expression of non-functional mutants of Sar1-GTPase (Stefanic et al., 2009) indicating that ESV biogenesis, similar to genesis of the cis-Golgi cisterna, may depend on the formation of COPII-associated carriers. Importantly, neither a steady-state stacked Golgi apparatus nor another stable delay compartment for
secretory cargo has been identified in this eukaryotic parasite, although trophozoites secrete constantly (Prucca and Lujan, 2009). Giardia trophozoites maintain a dense surface coat composed of one of ~200 variant-specific antigens (VSP) (Prucca et al., 2011; Nash et al., 1990). Transmembrane-anchored VSPs are continuously released into the environment and replaced by newly synthesized protein exported via a direct pathway from the ER to the plasma membrane. This phenomenon occurs alongside constitutive secretion of non-VSP proteins (Touz et al., 2011), indicating that secretory cargo is trafficked from the ER to distal compartments in the absence of an identifiable intermediate Golgi-like compartment for protein sorting and maturation. This observation complements the lack of complex N-glycosylation in Giardia (Samuelson et al., 2005), a common eukaryotic post-translational modification, suggesting that non-glycosylated proteins could be trafficked directly from their point of synthesis (the ER) to their destination.

However, the absence of a bona fide Golgi apparatus in Giardia calls for the presence of alternative sorting and cargo enrichment mechanisms which may rely entirely on the ER, more specifically on ERES. Our group previously reported on the concomitant deposition of CWP1 and of a constitutively-secreted plasma membrane marker at specific ER sites (Marti et al., 2003a). This preliminary characterization demonstrated how ESV-targeted reporters appear to be sorted from directly-secreted reporters before reaching ESVs, presumably at ER-related locations suggestive of ERES (Marti et al., 2003a). This supports the hypothesis that sorting of cargo in Giardia already occurs at the level of ER export in the absence of a Golgi platform, suggesting the presence of specialized ERES However, robust markers for the non-equivocal identification of these ER subdomains in Giardia had not yet been developed.

ERES have always shown to be in close connection to the Golgi apparatus. In Arabidopsis (Faso et al., 2009), yeast (Bevis et al., 2002) and the protozoan Trypanosoma brucei (Bangs, 2011; He et al., 2004), cis-Golgi cisternae are juxtaposed to ERES domains, while in mammalian cells the Golgi complex communicates with the surrounding ER by means of a vesiculo-tubular compartment (Reynaud and Simpson, 2002). The absence of a steady-state Golgi in Giardia trophozoites leads to the hypothesis that stable ERES are not present and that export of secretory cargo in trophozoites occurs in a random and sporadic fashion on the smooth ER surface, with no concentration at specific ER locations. In other words, organized ERES may have been lost during reductive evolution along with the Golgi. On the other hand, in trophozoites undergoing encystation, induction of a completely new secretory pathway which requires additional sorting and quality-
control capacity may presuppose formation of ERES as a prerequisite to the neogenesis of dedicated ESV delay compartments. ERES in trophozoites and/or encysting cells would indicate that these structures are able to mediate key functions in secretory cargo trafficking, such as sorting, independently of a stable Golgi apparatus and may to a certain extent still do so in organisms presenting Golgi (Mayor and Riezman, 2004; Watanabe and Riezman, 2004). *G. lamblia* is a unique and interesting model of choice for testing these hypotheses since this organism naturally lacks a classical Golgi apparatus but induces neogenesis of minimized cisternae during a defined developmental process and therefore does not require (potentially traumatic) mechanical manipulations to isolate Golgi-depleted cells (Tangemo et al., 2011; Pelletier et al., 2000).

Here we use *G. lamblia* to address the question whether secretory protein trafficking in the absence of a steady-state Golgi requires defined and stable ERES. For this, we developed tagged constructs for the expression of giardial homologues of well-characterized ERES and COPII components. This allowed us to identify and monitor the presence, dynamics of ERES in trophozoites and the relation to ESV biogenesis in encysting cells. We documented ERES and ESV behavior in proliferating cells and during encystation and could show that nascent ESVs are closely associated to ERES, similar to Golgi biogenesis in organisms with multiple Golgi. Furthermore, we could prove that disruption of ESV neogenesis by non-functional Sar1-GTPase mutants (Stefanic et al., 2009) is due to the absence of intact ERES, thereby adding to the body of data that supports ESVs as evolutionarily-reduced inducible Golgi analogues nucleated from ERES. Finally, we used fluorescence recovery after photobleaching (FRAP) analysis to investigate GlSec23 recruitment dynamics to ERES in both non-differentiating and encysting parasites as a read-out of ERES activity. Taken together, our work indicates how ERES can exist independently of permanent Golgi compartments and raises questions concerning Golgi-independent protein secretion mechanisms that remain to be elucidated in more complex eukaryotic systems.
Results

A giardial homologue of Sec23 localizes to punctae and to the cytosol in fixed and living cells

Using bioinformatics-based approaches, we identified a putative giardial Sec23 homologue encoded by ORF GL50803_9376 with a predicted size of 861 amino acids. BLAST-based searches of the Giardia Genome Database (GGD; www.giardiadb.org) using Sec23 homologues from several species as query sequences all returned ORF GL50803_9376 as the best hit, with e-values ranging from 5.9e-82 (Saccharomyces cerevisiae) to 4.4e-126 (Drosophila melanogaster) (Marti et al., 2003b). We further substantiated this initial identification by using the SMART set of algorithms to analyze the domain structure of the predicted candidate protein. ORF GL50803_9376 encodes for a protein product presenting all domains found in Sec23 homologues, namely the zinc finger Sec23-Sec24 domain (predicted with an e-value of 1e-9), the Sec23 trunk domain (predicted with an e-value of 7.80e-30), the Sec23 helical domain (predicted with an e-value of 5.1e-18) and the gelsolin domain (predicted with an e-value of 5e-3). Alignment of GlSec23 with several Sec23 homologues shows a good level of amino acid sequence conservation, with sequence identities ranging from 31 to 34% (data not shown). Therefore, the data derived from our in silico approaches strongly suggest that ORF GL50803_9376 encodes the giardial homologue of Sec23 (hereafter referred to as GiSec23).

As subunits of the COPII vesicle coat, Sec23 proteins are either present in a soluble form in the cytoplasm or incorporated into COPII coats of small membrane carriers and at specialized exit site domains at the smooth ER (ERES) where such carriers are formed in large quantities. We tested the subcellular localization of the giardial Sec23 to understand whether GiSec23 localizes to ER domains reminiscent of ERES. Trophozoites were transfected with a construct (expression vector CWP1p-HASec23HA, line Sec23HA) for the inducible expression of dually HA epitope-tagged GiSec23 (Sec23HA). Expression was induced for 12 hours; following cell fixation and detection of the HA tag with a fluorescently labeled monoclonal antibody (MAb), we observed 20-40 strongly-labeled distinct punctae throughout the cells in addition to a weak and diffused HA signal in the cytosol (Fig. 1A). Single-tagged GiSec23 variants distributed with an identical pattern (data not shown). This observation for Sec23HA accumulation is consistent with Sec23 localization in all eukaryotes analyzed to date. Importantly, Sec23-labeled punctate structures in other eukaryotic species have been shown to correspond to
ERES (Esaki et al., 2006; Stephens, 2003). This data suggests that ERES could be present in *G. lamblia* despite the absence of a stacked Golgi apparatus.

To investigate on the nature of the putative giardial ERES and on their relationship to the ER, we co-labeled fixed cells overexpressing Sec23HA for both the HA epitope and the endogenous ER membrane marker protein disulfide isomerase 2 (GlPDI2). The HA signal distribution at ERES showed a distinct association with the ER structure as defined by GlPDI2 (Fig. 1C), supporting an association of both structures.

To observe GlSec23 dynamics in vivo and to corroborate the data on Sec23HA localization, we cloned GlSec23 as a fusion to GFP, downstream of a 78bp fragment corresponding to the deduced endogenous promoter (expression vector Endop-Sec23GFP) to achieve constitutive expression of GlSec23-GFP (episomally-transfected line Endop-Sec23GFP). Wide-field live-cell imaging of this transgenic line demonstrated weak GlSec23-GFP signal in the cytosol and strong accumulation at scattered punctae, with a distribution analogous to that of Sec23HA in fixed cells (Fig. 1B). Taken together, the data are consistent with the conclusion that GlSec23 distributes in a cytosolic pool and is recruited to specific areas of the ER membrane, presumably ERES in both living and fixed cells. In both cases GlSec23 expression did not appear to have any discernible effect on cell growth, viability and trophozoites’ ability to differentiate (data not shown).

**Co-detection of epitope-tagged GlSec23 and a giardial Sec24-like protein homologue at putative ERES**

Sec23 has been consistently shown to interact with Sec24 to form the inner layer of a COPII vesicle (Haucke, 2003). For this reason, Sec24 has frequently been used as an additional marker for ERES. Therefore, to further validate our observations for GlSec23-mediated ERES labeling in Giardia, we searched the giardial genome for homologs of additional COPII subunits such as Sec24. The GGD presents 3 ORFs-GL50803_16520, 17065 and 17164- predicted to encode Sec24-related proteins (Marti et al., 2003b). Although these sequences are phylogenetically related to Sec24, they present either shortened or missing signature domains with respect to a consensus model for this COPII component in well-characterized eukaryotes. For further analyses, we selected ORF GL50803_17164 which we named GlSec24-like 1. Based on analyses with the SMART algorithm set, this ORF encodes a protein with all Sec24 signature domains (zinc finger Sec23-Sec24 domain: 1.3e-5; Sec23 trunk domain: 3.9 e-19; Sec23 beta sheet: 1.1e-9) except for the gelsolin domain, hereafter referred to as GlSec24-like 1. Co-expression of myc-tagged GlSec24-like 1 and
Sec23HA from a single transfected construct (expression vector 2p-mycSec24/1p-HASec23HA, line mycSec24-Sec23HA), followed by detection of both epitopes by immuno-fluorescence microscopy, demonstrated how both constructs mostly localized to the cytosol and to identical punctate structures, corresponding to putative ERES (Figs 2C, F). Shortly after induction of expression, a small number of these structures appeared to be labeled with either myc-tagged GlSec24-like 1 or Sec23HA (Fig. 2C).

Non-functional Sar1-GTPase abolishes giardial ERES assembly in differentiating trophozoites

Sar1-GTPase function is necessary for the recruitment of all subsequent COPII components to an ERES (Oka et al., 1991). Consequently, the expression of mutant Sar1-GTPase that binds GTP but is unable to hydrolyze it exerts a dominant-negative effect on COPII-dependent secretion in transgenic cells (Nakano et al., 1994). This effect is supposedly mediated by a loss of ERES functionality, leading to depletion of COPII-coated ER-derived membrane carriers. Due to this effect, conditionally expressed “GTP-locked” Sar1-GTPase mutants constitute a useful molecular tool to assess ERES functionality and trafficking of secretory proteins through these domains. We implemented this approach to test whether the punctate structures labeled by GlSec23 in fixed cells could be disrupted following expression of a Sar1-GTPase mutant. Furthermore, we monitored CWP1 accumulation to determine the impact of mutant Sar1-GTPase expression on the trafficking of differentiation-specific (CWP1) secretory protein cargo. For these purposes, we transfected giardial cells with a single inducible plasmid vector for the co-expression of Sec23HA with either wildtype giardial Sar1-GTPase (ORF Gl50803_7569; expression vector 2p-mycSar/1p-HASec23HA, line mycSar-Sec23HA) or a mutant Sar1-GTPase (H74G; expression vector 2p-mycSarmut/1p-HASec23HA, line mycSarmut-Sec23HA) previously shown to inhibit encystation in a dominant-negative fashion (Stefanic et al., 2009). In both cases Sar1-GTPase was tagged at the N-terminus with a myc-derived epitope. We verified correct expression of both wildtype and mutant Sar1-GTPase in separate fluorescence microscopy experiments (Figs 1SA-D).

We monitored Sec23HA accumulation in these cell lines as a measure of ERES integrity and compared it to Sec23HA localization in a cell line with no additional Sar1-GTPase-encoding ORFs. Expression of Sec23HA alone did not influence cell growth (data not shown) or ESV formation (Figs 3A-C). Similarly, analysis of line mycSar-Sec23HA co-expressing wildtype Sar1-GTPase and Sec23HA showed no discernible perturbation of either ESV formation (Stefanic et al., 2009) or GlSec23 accumulation at the punctate structures (Figs 3D-F).
both lines, CWP1 was detected at the periphery of ESVs, consistent with the partitioning of cyst wall proteins in maturing ESVs (Konrad et al., 2010). However, when the mutant Sar1-GTPase was expressed, the specific localization of Sec23HA at the punctate structures was lost and the Sec23HA reporter appeared almost exclusively dispersed in the cytosol (Figs 3G-I). In contrast to cells from lines Sec23HA and mycSar-Sec23HA, cells expressing the mutant Sar1-GTPase failed to form ESVs with condensed cores (compare Figs 3C,F-I), although CWP1 was detected at abundant levels by fluorescence microscopy. Interestingly, CWP1 in these cells showed a completely atypical distribution in elongated cisternae of the ER as well as in the nuclear envelope (Fig. 3H, inset).

Dissolution of the punctate structures labeled by the GlSec23 reporter in transgenic cells expressing a mutant Sar1-GTPase presents a strong argument for defined ERES in Giardia. The dependence of ESV formation on ERES maintenance in encysting cells after CWP export from the ER is consistent with our model whereby ESVs, similar to the Golgi apparatus (Ward et al., 2001), depend on functional ERES for their biogenesis.

**ERES presence and association to ESVs in vegetative and differentiating parasites**

Our working model predicts that ESV formation depends directly on giardial ERES, in accordance with the fundamental role played by ERES in Golgi biogenesis (Marti et al., 2003b). Our data depicting the inhibitory effect of mutant Sar1-GTPase on ERES maintenance and ESV formation confirms an important implication of our hypotheses concerning the Golgi-like nature of ESVs. To be able to investigate ERES distribution as well as the spatio-temporal association between ERES and ESVs in trophozoites and differentiating cells, we expressed the GlSec23-HA reporter constitutively by cloning the GlSec23 ORF downstream of the same 78bp endogenous promoter fragment used previously for construct Endop-Sec23GFP (expression vector Endop-Sec23HA, episomally-transfected line Endop-Sec23HA). With construct Endop-Sec23HA we achieved a distinctly detectable level of HA-tagged GlSec23 expression in trophozoites which allowed us to assess the number, behavior and association to ESVs of ERES in trophozoites and differentiating parasites.

**ERES number in vegetative and differentiating cells**

To understand whether the overall number of ERES in Giardia is influenced by the large increase in secretory flux associated with the export of CWPs during the encystation process, we sought to quantify ERES in
proliferating trophozoites and in cells harvested at 4 specific stages of differentiation as defined in (Konrad et al., 2010):

1) Neogenesis phase: CWP1 was detected exclusively in the ER and at knob-like accumulations directly associated with the ER. This stage corresponded to cells harvested up to 2-3 hours post induction of differentiation (hpid).

2) Accumulation phase: CWP1 was almost exclusively detected in enlarging ESVs that have not yet sorted their cyst protein material. Cells harvested at this stage were encysted for 6-7 hpid.

3) Partitioning phase: CWP1 was almost exclusively detected at the periphery of mature ESVs which contain a condensed core, indicating that CWPs present in these organelles had been processed and partitioned in preparation for sorting and secretion. This stage of encystation is representative for 8-14 hpid.

4) Cyst: CWP1 was detected exclusively in the cyst wall of differentiated parasites after sorting and rapid secretion from 14-16 hpid onward.

Based on the encystation categories thus defined, we asked whether de novo generation of ESVs would be either preceded and/or paralleled by a significant increase in ERES in encysting cells, compared to non-differentiating trophozoites. To test this, we acquired z-series image stacks of ≥ 50 cells for every encystation category, including non-encysting trophozoites and, following 3D reconstructions, we counted ERES in each stack (Fig. 4A).

Our data strongly suggests that the induction of ESVs is not accompanied by a substantial increase in the absolute number of ERES (Fig. 4B). We detected between 44 and 47 ERES/cell (average standard deviation 9.67) in trophozoites and the three first stages of differentiation. Statistical analysis (2-tailed Student’s t-test) indicated that the minor differences recorded between time-points are not significant (p>0.05). This data suggests that ERES in non-differentiating parasites are capable of accommodating the large quantity of secretory material that marks the beginning of encystation and the neogenesis of ESV compartments, without the need for an increase in their number. This is strikingly dissimilar to previously characterized eukaryotic systems such as plant and insect cells, where the need for increased secretory capacity is usually mirrored by amplification of both Golgi organelles and ERES (Farhan et al., 2008; daSilva et al., 2004; Stephens, 2003). Interestingly, we recorded the most significant changes in overall ERES numbers in cysts, where we observed...
2 subpopulations, one with ca. 45 ERES/cell and the other with ca. 4 ERES/cell (Fig. 4A, cysts). This data suggests that, despite its seemingly quiescent metabolic state (Svard et al., 1998) a terminally differentiated giardial cell maintains a certain degree of secretory capacity. Furthermore, it suggests that ERES numbers may reflect different stages of cyst maturation (Konrad et al., 2010).

At all stages of differentiation, we observed that not all ERES appeared to be labeled with the same intensity. However, a preliminary inspection of the labeling intensities of ERES in the image stacks did not reveal any evident differences between encystation time points (data not shown). Taken together with the determined ERES number per cell, this suggests that export of the large quantities of cyst wall proteins from the ER to ESVs requires neither an increase nor an enlargement in these ER subdomains.

**ERES association to nascent and maturing ESVs**

Based on the distinct perturbation of ERES and ESV formation in the presence of a dominant-negative Sar1-GTPase mutant, we hypothesized that ESVs arise not as mere subdomains of the giardial ER and that ESV neogenesis depends on functional ERES. This could mean that ESVs are always in close proximity to ERES, at least during the earlier stages of their formation. To test whether a physical association between ERES and ESVs can be observed at any time during differentiation, cells used for ERES quantification were also labeled with an anti-CWP1 antibody to assess the spatial association of ERES and CWP1/ESVs during encystation. Our fluorescence microscopy data indicates that ERES labeled with GlSec23-HA and the first accumulations of CWP1 in ER-associated knob-like structures typically detected at ~2 hours post induction of differentiation (hpi) overlap with high probability (Fig. 5A). This observation was further supported by transmission electron microscopy imaging of early-encysting *G. lamblia* cells where CWM-containing pre-ESV compartments were clearly visible as subdomains enveloped by ER membranes (Fig. 5B). However, already at ~3 hpi, nascent ESVs become clearly defined and normally show no direct association with ERES anymore, as seen in a loss of signal overlap (Fig. 5C). This spatial disassociation is maintained throughout the later stages of ESV growth and maturation.

To investigate whether ERES are also involved in trafficking of constitutive secretory cargo, we episomally co-transfected Giardia cells with vector CWP1p-HASec23HA and with an expression vector for the inducible expression of the *Toxoplasma gondii* SAG1 exodomain fused to the *G. lamblia* variant surface protein H7...
transmembrane anchor followed by the pentapeptide CRGKA (construct SAG1-VSPct; Marti et al., 2002). In accordance with published reports, we mostly detected SAG1-VSPct at the plasma membrane (Marti et al., 2002). Additionally, we observed SAG1-VSPct deposition at punctate structures (Fig. 1SE) which were co-labeled by Sec23HA (Fig. 1SF). This data suggests that, similar to CWP1, intermediate trafficking of VSP derivatives such as construct SAG1-VSPct is also associated to ERES (Marti et al., 2003a).

High-resolution detection of GI Sec23 using cryo-immuno electron microscopy

ERES as detected in immuno-fluorescence microscopy appear as small punctae but are likely below even the theoretical resolution of confocal microscopy. To investigate the morphology of giardial ERES in more detail, we sought to detect HA-tagged GI Sec23 deposition at a higher degree of resolution by performing immuno-electron microscopy (IEM). The HA epitope was detected by a specific MAb followed by a secondary anti-rat antibody coupled to 12nm gold particles. Using a cryo-sectioning protocol to increase membrane preservation, we could visualize Sec23HA accumulation at scattered sites either proximal to or directly on endomembranes (Fig. 6A-B, lower magnification in fig. 6C). Post-acquisition measurement of the distance between 175 individual gold particle labels and the closest visible membranous remnant demonstrates enrichment for Sec23HA in close proximity to an internal membrane (Fig. 6D). 61% of all gold particle labels were found either directly at a membrane remnant or within 20 nm from a membrane residue; 18% were found within a 50 nm distance while only 21% appeared either further away than 50 nm from the closest membrane in the section or were not proximal to any membrane. It is important to note that good membrane definition is difficult to achieve due to the high abundance of stained glycogen in the giardial cytosol. Therefore, we may have likely underestimated the number of gold particles associated to an internal membrane. Nevertheless, our data is consistent with observations in ERES EM characterization studies conducted on other eukaryotes (Ivan et al., 2008; Zeuschner et al., 2006) and is in good agreement with the general subcellular distribution of GI Sec23 which is either recruited to ERES or remains in the cytosolic pool. However, unlike previous accounts in other eukaryotes describing cup-shaped ER-derived structures corresponding to ERES (Hughes et al., 2009), we could not assign a specific morphology to the areas of Sec23HA deposition under these experimental conditions.
GlSec23-GFP shows high rates of exchange between ERES and the cytosolic pool

By implementing our construct for the expression of GFP-tagged GlSec23 (Endop-Sec23GFP), we used fluorescence recovery after photobleaching (FRAP) techniques to investigate GlSec23 recruitment dynamics to ERES as a measure of ERES turnover in both non-differentiating and encysting parasites, as a measure of ERES activity (Fig. 7). We did not detect significant differences in fluorescence recovery of ERES between the 2 parasitic populations, in both cases reaching close to 100% fluorescence recovery after approximately 2 minutes (Fig. 7F). This observation indicates that giardial ERES are long-lived structures and that inducible CWM deposition during parasite differentiation does not significantly alter the rate of Sec23 recruitment to ERES. Although giardial ERES appeared to be immobile, the recorded parameters of recovery suggest that remodeling of these ER sub domains is occurring constantly, regardless of the parasite’s differentiation status. This data is in line with previously-described Sec13-GFP FRAP dynamics in yeast-based experiments which highlighted characteristics of stability, immobility and constant remodeling for tER (Hammond and Glick, 2000).
Discussion

Giardia species present us with a striking degree of simplification in subcellular organization and endomembrane compartments. It may therefore seem unorthodox to use this organism as a tool to test the validity of ERES to Golgi correspondence model, described in all well-characterized eukaryotes. On the one hand, proliferating *G. lamblia* trophozoites present no discernible stable Golgi body, neither stacked nor dispersed. On the other hand, distinct Golgi-like delay compartments which contain only CWM but exclude all other secretory cargo are generated in differentiating trophozoites. For this reason, Giardia is uniquely suited to determine whether a cell which is naturally devoid of a permanent Golgi body requires distinct sites for ER export of secreted proteins. The data presented in this work provide an alternative scenario with respect to the universal model of ERES to Golgi correspondence, illustrating that ERES exist independently of a stable Golgi and that they are involved in trafficking of both inducibly- and constitutively- expressed secretory protein cargo.

Based on our analyses of the localization of HA-tagged GlSec23, myc-tagged GlSec24-like 1 and GlPDI2 at similar punctate ER-associated sites, we hypothesized these structures to most probably be ERES. Impaired Sec23 recruitment to ERES in the presence of a Sar1-GTPase GTP-locked mutant provided direct evidence for this hypothesis. Furthermore, we could show that ERES ablation was clearly associated with aberrant trafficking of CWP1. As a result, we determined a causal link between ERES functionality and ESV biogenesis, consistent with the evidence for ESVs as inducible Golgi remnants which may have evolved beyond immediate recognition (Dacks et al., 2003). This is underscored by the close association of nascent ESVs with ERES, which strongly suggests that, as we postulated, ERES play an important role in ESV neogenesis. Although this is a valuable observation that sheds light on the origins of ESVs, it does not allow us to make any statements about the relationship between the ER as a whole and ESVs as they reach their final size and mature to become sorting-competent. Furthermore, the exact role for ERES in sorting and export of CWPs to growing ESVs remains to be determined. Our fluorescence microscopy analyses experiments show that ESV organelles become distinct entities within the secretory pathway relatively early (~3 hours) after induction of differentiation. There is currently no mechanistical explanation for the physical separation of ERES and more established ESVs at this point in ESV genesis. One possible scenario is that ESVs are nucleated by homotypic fusion of membrane carriers containing CWM and originating from ERES. This is consistent with
their initial close proximity, but it remains to be determined whether ERES are actually able to carry the full load of maximal CWM export or whether another more direct transport route takes over.

Importantly, we detected ERES in both vegetative and encysting cells. This finding indicates that ERES are required in the non-differentiating trophozoite. Furthermore, we showed that a SAG1-VSP chimera colocalizes at ERES with GlSec23. Together with the absence of a post-ER delay compartment for the reporter, this supports the hypothesis that *G. lamblia* employs direct pathways for the trafficking of proteins such as VSPs from the ER to the plasma membrane (Marti et al., 2003a). This data underscores the previously postulated role of ERES as sorting stations for secretory transport in Giardia: in differentiating parasites these ER subdomains are key in the selective accumulation of cyst wall cargo in ESV while maintaining VSP turnover at the plasma membrane.

Interestingly, we did not detect significant fluctuations either in ERES quantity or in ERES turnover, as measured by HA- and GFP- tagged GlSec23 detection, respectively, during the parasite’s life cycle. This is in marked contrast to reports in other organisms where ERES numbers may increase dramatically following secretory stress, i.e. overloading of the ER with large amounts of cargo (Farhan et al., 2008; Hanton et al., 2007). Our data suggests that ERES which are already present in the non-encysting trophozoite are able to accommodate and traffic the extra cargo represented by CWM produced during the early phase of parasite differentiation and ESV neogenesis. What could change is the protein composition at these sites to allow the trophozoite to gain competency for the selective trafficking of CWM. A comparative analysis of ERES protein composition in vegetative and differentiating cells will provide important information on changes (if any) in the molecular quality and quantity of these protein complexes throughout the parasitic cycle. Alternatively, direct ERES to ESV transport of CWM may only be required for nucleation of ESVs (see below).

There are numerous implications for the discovery of ERES in a Golgi-less eukaryote regarding other trafficking-related machinery in the early secretory pathway such as carriers for both anterograde and retrograde flow and tethering complexes. The question concerning the type of structure adopted for the transport of encystation-specific cargo is especially relevant. While our data does not directly contribute to the identification of the carrier which mediates protein transfer between ERES and ESVs or the plasma membrane, it highlights a pivotal role for COPII components from an early stage in CWM trafficking. Whether the second stage of ER to ESV trafficking, when the bulk of the cargo is moved is also dependent on transport

© 2012 Blackwell Publishing Ltd
intermediates involving COPII, remains to be determined. An intriguing alternative is that direct tubular membrane connections are established between ER and ESVs through which high-capacity cargo export can be maintained without imposing undue stress on the ERES machinery. The previously observed mobility of soluble CWPs between ESVs may also be explained by such connections which would effectively link ESVs directly (Stefanic et al., 2009). This hypothesis would also be in line with previous reports which show that carriers transporting cargo from the ER to the Golgi can be both COPII-dependent and -independent and are pleiomorphic, including vesicles and tubular extensions (Mironov et al., 2003).

Defining carrier types for CWM transport between ERES or other ER-derived compartments and nascent ESVs will provide important indications on the existence of specific tethering machinery for CWM enrichment at ESVs. In yeast and mammalian cells, the TRAPP1 complex has been shown to interact specifically with Sec23 for the tethering of COPII vesicles, supposedly “marking” the vesicle for fusion to either another vesicle or to the Golgi apparatus (Cai et al., 2007). This complex is composed of 7 fixed and 3 accessory subunits. There are 5 putative homologues for TRAPP1 components in the Giardia genomic sequence suggesting that, despite the absence of a permanent Golgi body, this complex may still function in the tethering of ER-derived carriers. Determining the spatial and temporal distribution of these proteins could yield valuable information on whether TRAPP1 components are associated to Golgi-like ESVs and whether ERES in non-differentiating trophozoites “communicate” with an alternative endomembrane compartment. This is turn would provide us with a more complete picture of the full array of secretory organelles and trafficking pathways present in the eukaryotic kingdom, thus contributing to the “evolving paradigm” of the eukaryotic secretory pathway (Lippincott-Schwartz, 2011).
Experimental procedures

Giardia cell culture, transfection and in vitro encystation

Trophozoites of the *Giardia lamblia* strain WBC6 (ATCC catalog number 50803) were grown under anaerobic conditions in 11 ml culture tubes (Nunc, Roskilde, Denmark) containing TYI-S-33 medium supplemented with 10% adult bovine serum and bovine bile according to standard protocols (Hehl et al., 2000). Encystation was induced using the two-step method as described previously (Hehl et al., 2000), by cultivating the cells for 44 hours in medium without bile and subsequently in medium with porcine bile at pH 7.85. Circular plasmid DNA of expression vectors was linearized at the *Swa*I restriction site (Stefanic et al., 2009) and 15 µg of digested DNA was electroporated into $5 \cdot 10^6$ freshly harvested trophozoites on ice using the following settings: 350V, 960µF, 800Ω. Linearized plasmids were targeted to the *G. lamblia* triose phosphate isomerase (GI-TPI) locus and integration occurred by homologous recombination under selective pressure of the antibiotic puromycin (Invivogen, Toulouse, France: 36.3 µg/ml) for 5 days. Following selection of resistant clones, transgenic cell lines were maintained and analyzed without antibiotic. Stable transfection of *G. lamblia* cells was confirmed by PCR on extracted genomic DNA (gDNA) (Fig. 1SG) using oligonucleotides 1440 (5’CAAGATACTACACTCGTTGTTAATGTG) and 1441 (5’GGGCTTGTACTCGGTGCCCAT). These primers were designed to yield a ca. 2000bp amplicon spanning the predicted GI-TPI integration site for pPACV-Integ-derived constructs. gDNA quality was assessed by including primers 1352 (5’ATGCCCTTTCCTGGTCT) and 1353 (5’GCCTTGTGATTGCTCGAAGA) in the same amplification reaction (expected product size ca. 1100bp). Alternatively, trophozoites were transfected with undigested expression constructs, maintained as circular episomes under selective pressure with puromycin.

Expression vector construction

For the inducible expression of tagged proteins in *G. lamblia*, a previously described vector pPacV-Integ was used which allows for the expression of fusion proteins with a N-terminal HA-tag under CWP1 promoter (CWP1p) control, following puromycin selection of transfected cells (Stefanic et al., 2009). This construct was further modified in our laboratory to yield expression vector p2. This vector allows for the simultaneous inducible expression of 2 ORFs, where 1 expression cassette is under cyst wall protein 2 (CWP2p) promoter regulation and ends with the CWP2 3’untranslated region (UTR) and the other is under CWP1p control and...
flanked by the CWP1 3’UTR. Construct CWP1p-HASec23HA was synthesized by amplifying GlSec23 (GGD: Gl50803_ 9376; Genbank: XM_001708553.1) from G. lamblia WB C6 genomic DNA using primers 1032 (5’GACTAGTTACCACTACGATGTCCCGACTACGCTGTTGAGGCGGCGGAGTCCTTTG) and 908 (5’CGTTAATTAACTACGCTAGTCTGGGACATCTGATGGGATCCCTTGTGGCACTAC, thus inserting an HA tag-encoding sequence at the ORF’s 3’ end. This fragment was subsequently cloned in vector p2, downstream of the CWP1p and 5’ HA tag-encoding sequence, resulting in a dually HA-tagged GlSec23 ORF under CWP1p regulation. Construct 2p-mycSec24/1p-HASec23HA was generated by amplifying GlSec24like-1 (GGD: Gl50803_17164; Genbank: XM_001707788.1) from genomic DNA using primers 1033 (5’GATGCACAACAAAACACTCATCTCAGAAGAGGATGTCAAGGAGGTATCCAGCG) and 1058 (5’GACTAGTTTAGCGAACCCTTGGGCGCCTAG), thus incorporating a myc-tag at the 5’ end of the GlSec24like-1 ORF. This fragment was then cloned in expression vector CWP1p-HASec23HA downstream of the CWP2p. We generated constructs 2p-mycSar/1p-HASec23HA and 2p-mycSarmut/1p-HASec23HA in a similar fashion. We amplified GlSar1-GTPase (GGD: Gl50803_7569; Genbank: XM_001708602.1) in its wildtype and mutated (H74G) versions from constructs developed previously (Stefanic et al., 2009) using primers 1052 (5’GATGCACAACAAAACACTCATCTCAGAAGAGGATGTCAAGGAGGTATCCAGCG) and 1053 (5’GCTCTAGATTTAGGGAGAGCCAC) to insert a myc-tag at the 5’ of the GlSar1-GTPase ORFs. Fragments mycSar and mycSarmut were then separately cloned downstream of the CWP2p in construct CWP1p-HASec23HA to obtain expression vectors 2p-mycSar/1p-HASec23HA and 2p-mycSarmut/1p-HASec23HA, respectively. Expression of Sec23HA in all constructs described above was confirmed by immunoblotting (data not shown).

To generate expression vector Endop-Sec23HA, we amplified a 78bp putative endogenous promoter fragment followed by GlSec23 from genomic DNA using primers 1161 (5’CTCTAGAACAAGGTGCCCTGC) and 908 (5’CGTTAATTAACTACGCTAGTCTGGGACATCTGATGGGATCCCTTGTGGCACTAC), thus incorporating an HA tag-encoding sequence at the ORF’s 3’ end, yielding fragment Endop-Sec23HA. The 78bp putative endogenous promoter fragment lies between the end of the ORF preceding GL50803_9376 and the translation-initiation codon of GL50803_9376. The corresponding sequence is shown below with the ATG codon in bold:
In line with previous reports (Elmendorf et al., 2001) we could identify a transcription initiation site (underlined) characterized by AT-rich elements, spanning position -11 to position -2. Fragment Endop-Sec23HA was subsequently cloned in a standard pPacV-Integ with no additional HA tag-encoding sequences. Expression vector Endop-Sec23GFP was synthesized by amplifying GlSec23 from genomic DNA using primers 1161 and 977 (5' CTCCCTGACGGGCTTCTCCTGTTGCACTAC). The resulting fragment was subcloned in a previously developed vector (Hehl et al., 2000) to obtain an in-frame 3' fusion to a GFP-encoding ORF. The full-length fragment consisting of the deduced GlSec23 promoter fragment, followed by GlSec23-GFP, was cloned in a standard pPacV-Integ with no additional epitopes. Synthesis of expression vector SAG1-VSPct was described previously (Marti et al., 2002).

Cell imaging techniques

Immuno-fluorescence analysis

Chemical fixation and preparation for fluorescence microscopy was performed as described (Marti et al., 2003a). Briefly, cells were washed with cold PBS after harvesting and fixed with 3% formaldehyde in PBS for 40 min at 20°C, followed by 5 minutes incubation with 0.1 M glycine in PBS. Cells were permeabilized with 0.2% triton X-100 in PBS for 20 min at room temperature and blocked overnight in 2% BSA in PBS. Incubations of all antibodies were done in 2% BSA/0.2% Triton X-100 in PBS for 1 h at 4°C. The following antibodies were used in this work: FITC-conjugated and unconjugated anti-HA epitope (Roche Diagnostics GmbH, Manheim, Germany; dilution 1:50), Texas Red-conjugated anti-CWP1 (Waterborne™, Inc., New Orleans, LA, USA; dilution 1:80), anti-SAG1 DG52 (dilution 1:1000) (Bulow and Boothroyd, 1991), anti-giardial GlPDI2 (produced in our laboratory; dilution 1:500), anti-myc epitope (Sigma, Buchs, Switzerland; dilution 1:200), Alexa594-conjugated anti-mouse (Invitrogen, Eugene, OR, USA; dilution 1:300) and Alexa488-conjugated anti-rat (Invitrogen, Eugene, OR, USA; dilution 1:200). Post incubation washes were done with 1% BSA/0.1% triton X-100 in PBS. Labeled cells were embedded for microscopy with Vectashield (Vector Laboratories, Inc., Burlingame, CA, USA) containing the DNA intercalating agent 4'-6-Diamidino-2-phenylindole (DAPI) for detection of nuclear DNA. Immuno-fluorescence analysis was performed on a Leica
SP2 AOBS confocal laser-scanning microscope (Leica Microsystems, Wetzlar, Germany) equipped with a glycerol objective (Leica, HCX PL APO CS 63x 1.3 Corr). Confocal image stacks were recorded with a pinhole setting of Airy 1 and twofold oversampling. Further processing was done using the Huygens deconvolution software package version 2.7 (Scientific Volume Imaging, Hilversum, NL). Three-dimensional reconstructions and quantitative analysis of co-localization were done with the Imaris software suite (Bitplane, Zurich, Switzerland). Alternatively, a standard fluorescence microscope (Leica DM IRBE) and MetaVue software (version: 5.0r1) was used for data collection and image processing. To generate 3D reconstructions of labeled ERES, a standard fluorescence microscope (Leica DM IRBE) was used to acquire stacks consisting of 30-35 images taken at a distance of 0.2 μm between subsequent planes, starting from the plane of the last visible ERES and then spanning the whole cell (ventral to dorsal). Projection views of the stacks were generated using the MetaVue software (version: 5.0r1) and ERES within entire cells were counted.

Live cell microscopy and fluorescence recovery after photobleaching (FRAP)

For live cell microscopy, non-differentiating or 12 hpid cells constitutively expressing the GlsSec23-GFP chimera were harvested and transferred to 24-well plates at a density of 6·10⁶/ml. After incubation on ice for 16 h, oxygenated cells were sealed between microscopy glass slides and warmed to 37°C. For FRAP, and time-lapse series, images were collected on a Leica SP2 AOBS confocal laser-scanning microscope (Leica Microsystems, Wetzlar, Germany) using a 63x water immersion objective (Leica, HCX PL APO CS 63x 1.2 W Corr). Fluorescence in selected regions of interest was quantified using the corresponding Leica software suite. The pinhole was set to Airy 2 in order to increase the thickness of the optical sections to accommodate an entire ESV in the z-plane. Quantifiable criteria for cell viability were active attachment to substrate and continuous beating of the ventral and anterolateral flagella pairs. FRAP experiments were performed using previously reported settings (Stefanic et al., 2009) with Leica FRAP software module to set bleaching parameters and to quantify fluorescence recovery.

Electron Microscopy Techniques

- Transmission electron microscopy
Samples were prepared as previously described (Marti et al., 2003a). Trophozoites and encysting parasites were grown on 30-µm-thick sapphire disks coated with a 5-nm carbon film. The disks, containing a single layer of attached parasites, were plunged into a mixture of liquid propane/ethane (8/2) cooled by liquid nitrogen. The ultrarapidly frozen samples were substituted at −90°C in acetone containing 0.5% osmium tetroxide and 0.25% glutaraldehyde (Wild et al., 2001 blue right-pointing triangle) overnight. The temperature was then continuously (5°/h) raised to 0°C, and the samples were embedded in epoxy resin at 4°C. After polymerization at 60°C for 2 d, ultrathin sections were cut parallel to the sapphire surface, stained with uranyl acetate and lead citrate, and examined in a CM 12 electron microscope (Philips, Eindhoven, Netherlands) equipped with a slow-scan CCD camera (Gatan, Munich, Germany) at an acceleration voltage of 100 kV. Recorded pictures were processed with the Digital Micrograph 3.34 software (Gatan).

• Cryo-Immuno Electron Microscopy

Samples was prepared according to the Tokuyasu method (Tokuyasu, 1973). In brief, cells were fixed with 4% formaldehyde and 0.2% glutaraldehyde for 2 hours at room temperature, washed several times with 0.1M Na-phosphate pH 7.4, and pelleted at 37°C in 12% gelatin. The gelatin-embedded blocks were immersed in 2.3 M sucrose kept overnight at 4°C, mounted on specimen holders of an ultramicrotome (UC6, Leica Microsystems, Wetzlar, Germany) and frozen by plunging into liquid nitrogen. After trimming to suitable block size and shape, 70 nm sections were cut at -120°C using a dry diamond knife (Diatome, Biel, Switzerland). Flat ribbons were picked up with a wire loop filled with a drop of 1% methyl cellulose and 1.15M sucrose in 0.1M Na-phosphate, pH 7.4, and transferred onto carbon-coated formvar films mounted on 100 hexagonal mesh/inch copper grids. For immuno-labeling, sections were washed with 50mM glycine, blocked with 1% BSA and incubated with rat-derived anti HA (Roche Diagnostics GmbH, Manheim, Germany) at a dilution of 1:5 at room temperature for 90 minutes, washed several times with 0.1% BSA, incubated with goat anti rat antibodies coupled with 12nm colloidal gold (Jackson ImmunoResearch Laboratories, Inc. West Grove, PA, USA), washed with 0.1M Na-phosphate pH 7.4 and distilled water, and transferred to a mixture of 1.8% methyl cellulose and 0.4% uranylacetate. After 5 minutes, the grids were looped out and excess solution was drained away. After air drying a thin film remained over the grid. The sections were analyzed in a transmission...
electron microscope (CM 12, Philips, Eindhoven, The Netherland) equipped with a CCD camera (Ultrascan 1000, Pleasanton, CA, USA) at an acceleration voltage of 100 kV.

Acknowledgements

We are thankful to Therese Michel for excellent technical support and to Cornelia Spycher for the development of vector p2. We are grateful to our co-workers and to the reviewers of this paper for their valuable feedback. This project was funded by Swiss National Science Foundation Grant 31003A_125389 awarded to ABH. The authors declare no competing interests.
Figure legends

Figure 1 GiSec23 localizes to ER-associated punctate structures

(A) Detection of dually HA-tagged GiSec23 in encysting cells of line Sec23HA after 12 hours induction of expression, employing wide-field fluorescence microscopy. Aside from weak cytosolic deposition, Sec23HA mainly localized to punctate structures dispersed throughout the giardial cell. Inset: bright-field image showing ESVs with condensed cores. Scale bar: 1 µm.

(B) Wide-field live-cell imaging of non-differentiating trophozoites from line Endop-Sec23GFP constitutively expressing a C' terminal GiSec23-GFP fusion. Similarly to Sec23HA localization in fixed cells, GiSec23-GFP was detected in the cytosol and at discrete punctate structures. Inset: bright-field image. Scale bar: 1 µm.

(C) Representative subcellular distribution of Sec23HA (green) in a transgenic trophozoite from line Sec23HA labeled with anti-GIPDI2 antibodies (ER membranes, red); images were acquired with confocal laser-scanning fluorescence microscopy. Nuclei were labeled with DAPI (blue). Insets: bright-field image and enlarged area (merged image). Scale bar: 2 µm.

Figure 2 Myc-tagged GiSec24 like-1 and HA-tagged GiSec23 co-localize at putative ER exit sites

Wide-field fluorescence microscopy for the detection of myc-tagged GiSec24 like-1 (red) and Sec23HA (green) deposition in a representative trophozoite from cell-line mycSec24-Sec23HA after 4 hours (A-C) and 12 hours (D-F) induction of expression. Both epitopes resided at identical putative ERES structures. Nuclei were labeled with DAPI (blue). Insets: bright-field images. Scale bars: 1 µm.

Figure 3 Conditional expression of a Sar1-GTPase H74G mutant abolishes giardial ERES assembly in differentiating trophozoites

Wide-field fluorescence microscopy analysis of differentiating trophozoites co-expressing Sec23HA (green) with either wildtype myc-tagged GiSar1-GTPase (line mycSar-Sec23HA) or H74G mutated GiSar1-GTPase (line mycSarmut-Sec23HA) were labeled for Sec23HA and CWP1. Overexpression of Sec23HA alone did not affect either Sec23HA localization at putative ERES (A) or CWP1 deposition at the periphery of condensed ESVs (B). Similarly, overexpression of wildtype GiSar1-GTPase and Sec23HA did not perturb putative ERES (D) nor did it influence CWP1 localization (E). In both cases, brightfield images show accumulation of ESVs with condensed cores (C, F). In cell-line mycSarmut-Sec23HA, Sec23HA accumulation was clearly affected, with no detectable punctate structures and dispersed cytosolic signal (G). CWP1 was present in enlarged post-

© 2012 Blackwell Publishing Ltd
ER compartments (H, inset) and almost no condensed ESVs are detected in corresponding bright-field images (I). Nuclei were labeled with DAPI (blue). Scale bars: 1 µm.

**Figure 4** The overall number of ERES does not change significantly during parasite differentiation

(A) Cells from the line Endop-Sec23HA were sampled at 0 hours post induction of differentiation (hpid) and subsequently at 3-4, 6-7, 9-10 and 14-16 hpid. Following co-labeling for Sec23HA (green) and CWP1 (red), cells were imaged using wide-field fluorescence microscopy. For every timepoint, at least 50 cells were selected for wide-field z-stack acquisitions followed by 3D renditions of the Sec23HA signal at ERES. The selected cells are representative of each time point. Nuclei were labeled with DAPI (blue). Insets: bright-field images. Scale bars: 1 µm.

(B) Based on the 3D renditions shown in (A), average values for ERES/cell were calculated for every timepoint and plotted with their standard error (vertical bars).

**Figure 5** ERES and CWP1/ESVs are spatially associated in the very early stages of encystation

(A) Representative subcellular distribution of Sec23HA (green) in an encysting cell from line Sec23HA at 2 hours post induction (h. p.i) detected by confocal laser-scanning microscopy. The large majority of CWP1 accumulations (red) co-localized with ERES (inset merge). Nuclei were labeled with DAPI (blue).

(B) Transmission electron microscopy image showing CWM-containing pre-ESV compartments surrounded by ER membrane remnants during early stages of encystation (2h. pi.) in wild type *G. lamblia* cells. Inset: trace highlighting CWM (red) and ER membranes (blue).

(C) Representative subcellular distribution of Sec23HA (green) in an encysting cell from line Sec23HA at 3h p.i. acquired by confocal laser-scanning microscopy. Nascent ESVs are detectable and spatially separated from ERES (inset merge). Nuclei were labeled with DAPI (blue). Insets: bright-field images and enlarged areas (merged images). Scale bars: 2 µm.

**Figure 6** Detection of epitope-tagged GlSec23 by cryo-immuno electron microscopy

(A) and (B) High magnification electron microscopy image of gold particle labeling of Sec23HA in encysting cells from line Sec23HA. Several sites of Sec23HA association can be distinguished at visible membrane remnants (purple arrows). The nucleus is also marked (green star). Scale bar: 100nm.

(C) Low magnification electron microscopy image of an encysting cell from line Sec23HA, showing a nucleus (green star), 2 ESVs (red stars) and the adhesive disk (arrow). Scale bar: 2 µm.
(D) Gold label distribution of Sec23HA deposition in cells shown in (A). 61% of the label was detected in close proximity (0-20 nm) of a visible membrane remnant.

**Figure 7** Fluorescence recovery after photobleaching (FRAP) analysis of ERES labeled with GlSec23-GFP

(A) Regions of interest (ROIs 1-3) were defined in living trophozoites and ROI1 was photobleached. Qualitative (A-E) and quantitative (F) analysis of fluorescence recovery at the bleached ERES clearly indicated a significant turnover of GlSec23-GFP at these ER subdomains. (F) Quantification of fluorescence in ROIs 1-3: 2 pre-bleach scans (0-3 secs), 6 bleaching cycles (3-46 secs), 24 post-bleach scans (46-124 secs). Solid lines represent fluorescence levels $[F]$ in ROIs. Linear regressions for ROIs 1 and 2 are indicated. No data was collected during the bleach cycles: broken lines connect the last pre-bleach scan to the corresponding post-bleach scan. This data is representative of 5 cells selected for this analysis. Inset: bright-field image. Scale bar: 2 µm.

**Figure 1S**

**Myc-Sar-1GTPase detection in cell lines mycSar-Sec23HA and mycSarmut-Sec23HA**

Representative images for Sec23HA and Sar1-GTPase deposition in cell lines mycSar-Sec23HA and mycSarmut-Sec23HA, using wide-field fluorescence microscopy. Sec23HA accumulation at ERES is not affected by overexpression of wildtype Sar1-GTPase (A) while it is clearly disrupted in the presence of mutated Sar1-GTPase (C). Both wildtype (B) and mutant (D) myc-tagged Sar1-GTPase show a diffused localization pattern. Nuclei were labeled with DAPI (blue). Insets: bright-field images. Scale bar: 1 µm.

**A SAG1 construct carrying a VSP trafficking signal and Sec23HA co-localize at ERES**

(E) Representative image acquired by wide-field fluorescence microscopy of differentiating trophozoites expressing construct SAG1-VSPct. The SAG1 exodomain (red) was detected at the plasma membrane and at discrete intracellular sites reminiscent of ERES. Nuclei were labeled with DAPI (blue). Inset: bright-field image. Scale bar: 1 µm.

(F) Representative image of a differentiating trophozoite co-expressing SAG1-VSPct (red) and Sec23HA (green) at ERES, acquired by wide-field fluorescence microscopy. Nuclei were labeled with DAPI (blue). Inset: bright-field image. Scale bar: 1 µm.
Confirmation of Gl-TPI-directed chromosomal integration of pPACV-Integ-derived constructs in cell lines Sec23HA, mycSec24-Sec23HA, mycSar-Sec23HA and mycSarmut-Sec23HA. Genomic DNA samples from untransfected cells (lane 1) and from cell lines Sec23HA (lane 2), mycSec24-Sec23HA (lane 3), mycSar-Sec23HA (lane 4) and mycSarmut-Sec23HA (lane 5) all present the control amplicons (ca. 1100bp) derived from a non-targeted locus. However, only samples derived from stably-transfected lines (lanes 2-5) present the integration-derived ca. 2000bp amplicon, indicating that construct integration in gDNA of stably-transfected lines occurred within the Gl-TPI locus. M: GeneRuler DNA ladder marker (Fermentas); selected bandsizes in bp are indicated on the left.
Bibliography


© 2012 Blackwell Publishing Ltd


FIGURE 1
FIGURE 2

sec23HA  mycSec24  DAPI

early encystation

D  E  F

late encystation
FIGURE 4

A

Differentiation status
Sec23-HA
CWP1
DAPI

3D ERES rendition

hpid

No encystation
CWM in ER
Pre-partition ESVs
Post-partition ESVs
Cyst

0
3-4
6-7
9-10
14-16

B

average ERES/cell

hpid

0
3-4
6-7
9-10
14-16
FIGURE 7

A: Image showing ROI1, ROI2, and ROI3 with bars indicating measurements.
B: Pre-bleach image with ROI1 highlighted.
C: Bleach image showing ROI1.
D: Post-bleach 46s image with ROI1 highlighted.
E: Post-bleach 120s image with ROI1 highlighted.

Graph F shows the bleaching cycles with ROI1, ROI2, and ROI3 plotted over time.