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Recent advances in functional research in *Giardia intestinalis*

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Abstract

This review considers current advances in tools to investigate the functional biology of *Giardia*, it's coding and non-coding genes, features and cellular and molecular biology. We consider major gaps in current knowledge of the parasite and discuss the present state-of-the-art in its in vivo and in vitro cultivation. Advances in in silico tools, including for the modelling non-coding RNAs and genomic elements, as well as detailed exploration of coding genes through inferred homology to model organisms, have provided significant, primary level insight. Improved methods to model the three-dimensional structure of proteins offer new insights into their function, and binding interactions with ligands, other proteins or precursor drugs, and offer substantial opportunities to prioritise proteins for further study and experimentation. These approaches can be supplemented by the growing and highly accessible arsenal of systems-based methods now being applied to *Giardia*, led by genomic, transcriptomic and proteomic methods, but rapidly incorporating advanced tools for detection of real-time transcription, evaluation of chromatin states and direct measurement of macromolecular complexes. Methods to directly interrogate and perturb gene function have made major leaps in recent years, with CRISPr-interference now available. These approaches, coupled with protein over-expression, fluorescent labelling and in vitro and in vivo imaging, are set to revolutionize the field and herald an exciting time during which the field may finally realise *Giardia*'s long proposed potential as a model parasite and eukaryote.

1. Introduction

Undertaking comprehensive functional studies has remained a persistent obstacle in parasitological research, outside of a small number of apicomplexans, such as species of *Plasmodium* and *Toxoplasma gondii* (Limenitakis and Soldati-Favre, 2011; Meissner et al., 2007). Primary obstacles to genetically tractable parasites include an inability to readily culture them outside of a host, a lack of knowledge of the genetic and regulatory systems of

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parasites or the unavailability of tools to apply to them. *Giardia intestinalis* has been in vitro culturable for several decades in complex media, principally Keister's modified TYI-S-33 (Davids and Gillin, 2011) and its regulatory genetics and gene composition have been explored through the publication of reference genomes (Franzen et al., 2009; Jerlstrom-Hultqvist et al., 2010; Morrison et al., 2007) and other "omics" driven research over the past decade (e.g., Ansell et al., 2015a, 2017; Emery et al., 2018; Franzén et al., 2013; Ma'ayeh et al., 2018; Spycher et al., 2013), albeit with much still to be done. The complex binucleate and multiploidal (2N, 4N and 8N at various stages of the life cycle; Bernander et al., 2001) cellular biology of *Giardia* has proven a persistent obstacle to functional research, and for much of its post-genomic period efforts to develop functional tools for *Giardia* have met with limited success (Luján and Svärd, 2011). Because of its importance as a parasite, its scalable culturability in cell-free media, and its deep-branching position within the eukaryotic tree of life (Morrison et al., 2007), *Giardia* has long been proposed as an intriguing and potentially impactful model organism (Luján and Svärd, 2011), but its recalcitrance to genetic manipulation has proven a persistent road-block to realising this potential.

Here, we review advances in functional research of *Giardia*, drawing on recent publications in the field, as well as novel advances in other fields that may be applicable for *Giardia*. Our hope is that this review will act not only as a summary of the progress in research in this field over the last few years, but also as stimulus for renewed thinking on functional research in *Giardia* and on the potential to exploit these technologies for novel anti-giardial therapies, but to harness this fascinating protist as a model for eukaryotic biology.

2. Advances in in vitro cultivation

The inability to culture parasites in a laboratory setting, either in vitro or in vivo, presents the primary obstacle to advancing functional research. Fortunately, this obstacle has been overcome for *Giardia* for several decades. In vitro cultivation of *G. intestinalis* is readily undertaken in TYI-S-33 media (Davids and Gillin, 2011), with or without nitrogen-sparging for culture-adapted strains isolates. *Giardia intestinalis* trophozoites (at least of the assemblage A) can be triggered into in vitro cyst formation in encystation media, which is typically modified "high-bile" version of TYI-S-33 (Davids and Gillin, 2011).

Although *G. intestinalis* is a micro-aerophile and TYI-S-33 can be made anaerobic through nitrogen-sparging (i.e., gassing) or, in small volumes, using anaerobic pouches in a sealed container (Ansell et al., 2016), it is common practice to simply make media up in an aerobic environment, inoculate the culture with *Giardia* trophozoites and then seal the culture flask with a non-vented lid (Davids and Gillin, 2011). In this non-sparged approach, *Giardia* trophozoites may show a 24–48h lag-phase before exponential growth. Ansell et al. (2015a) showed that although trophozoites grow in the early 24–48h period, they transcribe many regulators of the oxidative stress response even up to 60h after inoculation and appear largely reliant on arginine metabolism for ATP. After ~60h in culture, these trophozoites dramatically shift their transcriptional behaviour, down-regulating their stress responses and up-regulating oxygen-sensitive glycolytic ATP production. Companion experiments in fully anaerobic media are needed, but these data suggest that culture conditions may have a

significant effect on the cellular behaviour of this oxygen-sensitive parasite and are worth considering in functional studies.

2.1 Systems for studies of *Giardia*-host cell interactions in vitro and in vivo

Giardia infects the upper small intestine and primarily interacts with intestinal epithelial cells (IECs) during host-cell adherence via its adhesive disc. However, it is difficult to grow primary IECs, and this has led to the use of intestinal cell lines as models in most in vitro studies of the small intestine (Dosh et al., 2018). Several in vitro models of host parasite-interactions during giardiasis have been developed using IECs in co-incubations with axenic *Giardia* parasites. The best described and most used cell line in studies of IECs is Caco-2, an adenocarcinomal colonic epithelial cell line that is restricted in propagation by cell-to-cell contact and can be induced to express small intestinal features (such as apical to basolateral polarization, formation of tight junctions and the appearance of microvilli) by growth post-confluence for around 20 days (Sambuy et al., 2005). Proteomic analyses have shown similar expression profiles between Caco-2 cells and cells derived from scrapings of the human intestinal epithelium (Lenaerts et al., 2007).

The Caco-2 cell line in its differentiated state is also the most used IEC in studies of *Giardia*-IEC interactions in vitro (Fisher et al., 2013; Kraft et al., 2017; Ma'ayeh et al., 2017, 2018; Roxstrom-Lindquist et al., 2005). The first studies were published in the early 1990s by Favennec et al. (1990, 1991) and mainly studied attachment and drug treatment, something that has been followed up in several other studies (Muller et al., 2006). The role of nitric oxide, studies of the intestinal barrier and several gene expression studies have been performed later (Eckmann et al., 2000; Ma'ayeh et al., 2018; Teoh et al., 2000). The human HT-29 cell line has also been used in *Giardia*-IEC interaction studies. It is more heterogeneous than the Caco-2 cell line, consisting mainly of un-differentiated cells and a smaller mucus producing subpopulation (3–5%) (Huet et al., 1987; Maoret et al., 1989). It can be differentiated into an enterocyte-like cell by glucose starvation, and several mucus-secreting clones have been established (Chastre et al., 1985; Lievin-Le Moal, 2013).

Other cell-lines for *Giardia* interactions include the HCT-8 cell line, which is derived from a human ileocecal adenocarcinoma and has been used in at least three studies of *Giardia*-host cell interactions (Koh et al., 2013; Panaro et al., 2007). The rat intestinal epithelial cells, IEC-6 have been used in several *Giardia* interaction studies and continuous co-culture, with purified giardial proteins and analysed from these interactions (Cabrera-Licona et al., 2017; Ma'ayeh and Brook-Carter, 2012; McCabe et al., 1991; Ortega-Pierres et al., 2018). A few studies of *Giardia*-host cell interactions have used the SCBN cell line, as it was initially described as a "small intestine epithelial cell line of human origin", but later studies showed that this cell line is of canine origin (Buret and Lin, 2008). However, the SCBN cell line does show different responses to *Giardia* parasites from different assemblages, and *Giardia* parasites are capable of both tight junction alteration and apoptotic induction and resistance in SCBN cells (Buret et al., 2002; Scott et al., 2002; Teoh et al., 2000).

The breadth of datasets from in vitro IEC-*Giardia* interaction models has highlighted several important processes during early pathogenesis (Einarsson et al., 2016c). Identification of key molecular mechanisms and pathways indicate multiple levels of crosstalk in the context of

virulence biology in the parasite, and immunology of the host. The in vitro data the community has generated to date, using simple in vitro systems, can lead to directed experiments in more complex human models like human small intestinal enteroids (Chen et al., 2017; Zachos et al., 2016). It should also be possible to use human biopsies from giardiasis patients in studies of specific factors and mechanisms.

In vivo model systems described below will also be important to verify the role of factors identified in vitro and to identify new factors and mechanism important during *Giardia* infections. *Giardia muris* has been used as a model for understanding the pathogenesis and immunological responses of the host during establishment of infection, beginning in the 1960s (Friend, 1966). The availability of knock-out mice and other host-related resources makes *G. muris* a powerful alternative model to study the induction of pathogenesis during *Giardia* infections, which cannot be accomplished by studying natural human infections or using human cell lines (Fink and Singer, 2017). The life cycle and infective process of *G. muris* is a close reflection of the infection by *G. intestinalis* (Dann et al., 2018). Several major findings of *Giardia* biology (flagellar and disc function and cellular differentiation) (Holberton, 1973; Schaefer et al., 1984) and immunity (IgA and Th17) responses, defensins and post-infectious colitis (Dann et al., 2018; Dreesen et al., 2014; Langford et al., 2002; Manko et al., 2017)have been pioneered in *G. muris*, and later been shown to be transferable to human *G. intestinalis* infections (Saghaug et al., 2016). Unfortunately, the research on *G. muris* has been constrained by the absolute lack of genome information and gene expression data, however, with a draft genome of *G. muris* is now available in public databases (NCBI and GiardiaDB), this should stimulate wider uptake of *G. muris* as an in vivo model.

3. Major knowledge gaps in functional biology in *Giardia*

Draft genomes have been available for *Giardia intestinalis* assemblage A (WB: Morrison et al., 2007), B (GS: Franzen et al., 2009) and E (P15: Jerlstrom-Hultqvist et al., 2010) for a number of years. These assemblies have provided the basis for predicting *Giardia*'s ~5500 coding gene models. Recent efforts (see current release in GiardiaDB) to curate these models have significantly improved their functional annotation; yet, ~60% of these are defined as "conserved" or "hypothetical" proteins. Additional work on completing the *G. intestinalis* genomes and refining the annotation of these coding genes (including through transcriptomic and proteomic support) are needed.

Regulatory regions of the *Giardia* genome are even less defined. Among coding models, neither promoters nor 5′ or 3′ untranslated regions (UTRs) are well characterized. To date, studies indicate that most coding *G. intestinalis* genes have little to no 5′ UTR and a greatly reduced 3′ UTR (Adam, 2000; Morrison et al., 2007), but this is not confirmed through broad-scale direct transcript sequencing (e.g., using PacBIO or Nanopore technology). There are few annotated transcription factors in the *G. intestinalis* gene set (Franzen et al., 2009; Jerlstrom-Hultqvist et al., 2010; Morrison et al., 2007), and their binding sites and the genes they regulate are largely unknown. To our knowledge, there has been no comprehensive study of enhancer regions in *Giardia*. Lastly, although *Giardia* shows significant variation in karyotype and chromatin condensation between nuclei (Tůmová et al., 2007) and during the cell-cycle (Tůmová et al., 2015) and histone methyltransferases appear to be important in

encystation (Carranza et al., 2016; Salusso et al., 2017; Sonda et al., 2010), antigenic variation (Carranza et al., 2016) and drug resistance (Ansell et al., 2015b), to date there has been no extensive study of chromatin level regulation in this parasite.

Post-transcriptional and post-translational regulation plays an important but largely unexplored role in *Giardia* biology. *Giardia intestinalis* was one of the first eukaryotes in which DICER was structurally resolved (MacRae et al., 2006). Consistent with this, *Giardia* has functional RNA interference systems (Prucca et al., 2008), but these have, as yet, been relatively unexplored. Small RNA sequencing has been undertaken in *G. intestinalis* WB trophozoites at confluence in TYI-S-33 media (Chen et al., 2009). This work characterised a large number of potential small-interfering and microRNAs, but did not define the genes regulated by them. To date, the only confirmed role for RNAi systems in *Giardia* is in regulation of variant-surface protein expression (Gargantini et al., 2016; Prucca et al., 2008; Saraiya et al., 2014). Studies of other post-transcriptional and post-translation regulatory systems are limited. Williams and Elmendorf (2011) showed evidence for Pumilio-domain (Puf) proteins in *Giardia*, which are RNA-binding proteins that play important post-transcriptional regulatory roles in a variety of eukaryotes (Gerber et al., 2004; Spassov and Jurecic, 2003). These have not been further explored.

Giardia has a reduced core kinome (Manning et al., 2011) as well as chromatin modifying enzymes including acetyltransferases, deacetyltransferase and methyltransferases (Iyer et al., 2008). In *Giardia* protein post-translational modifications of serine, threonine and tyrosine phosphorylationhas been demonstrated by Manning et al. (2011), ubiquitin by Niño et al. (2013) and SUMOylation by Vranych et al. (2014). Emery et al. (2018) provided evidence for widespread post-translational modifications (e.g., methylation) in metronidazole resistance, and Carranza et al. (2016) for roles of acetyllysine and methyllysine histone marks in *Giardia*, but broad-scale characterization of these modifications, particularly at site-specific levels, has not been undertaken.

4. Computational and “omic” approaches to functional biology research in *Giardia*

It is clear that the next major breakthroughs in our understanding of the molecular biology of *Giardia* must make full use of advances in functional research. Conceptually, these advances can be categorized into three major and complimentary themes: (i) improved in silico inference, (ii) direct empirical assessment of function through “omics”, labelling and other approaches and (iii) direct empirical assessment of function through targeted genetic manipulation.

4.1 In silico inference of function

Given its history as a promising but ultimately genetically intractable (or at best challenging) organism, much of what we currently understand about *Giardia*’s functional biology is based on in silico inference through comparative analyses with model organisms and other parasitic protists. In large part, this has restricted our understanding to the function of coding genes and is based primarily on BLAST homology with existing curated protein databases,

including the Kyoto Encyclopaedia of Genes and Genomes (KEGG: Kanehisa and Goto, 2000) and Uniprot (Consortium, 2014). However, although *Giardia*'s deep-branching origins in eukaryotes (Morrison et al., 2007) makes it an intriguing organism to study, the evolutionary distance between this parasite and other, more well characterized, eukaryotes is severely problematic. As noted above, nearly two-thirds of *G. intestinalis* coding genes lack an identifiable homologue in a genetically tractable organism.

Protein-domain modelling can assist in extracting additional functional information from these coding genes. Interproscan is the most well-known and widely used of these approaches (Jones et al., 2014). This method identifies conserved features of known protein domains from across eukaryotic and prokaryotic life, stored in a number of curated protein family databases, including InterPro (Mitchell et al., 2018), PANTHER (Mi et al., 2016) and pfam (El-Gebali et al., 2018). These conserved domain data can be used in combination to infer each protein's involvement in common biological functions and provided further functional context using the Gene Ontology Hierarchy (Consortium, 2016) or PANTHER (Mi et al., 2016). Hidden Markov Modelling (e.g., using HMMER: Potter et al., 2018) and other weighted matrix search strategies (e.g., Position Specific Information (PSI)-BLAST: Altschul et al., 1997) have added value for identifying homologous sequences for protein families or domains not covered in the InterProScan analysis. Whether searching curated or custom databases, in essence, these approaches rely on identifying proteins features that are widely conserved across evolutionary time. For the most part, these features are so conserved because of their importance in forming and maintaining the three-dimensional shape of the protein domain, which is crucial to its overall function.

Protein structure, which largely imparts function, generally evolves more slowly than protein sequence and can be determined by a small number of essential amino acid residues not readily identifiable through comparative sequence alignments. Noting this, protein structural homology can be used as a powerful, in silico method to infer protein function, even across large evolutionary distances. These approaches rely on modelling protein structure from the underlying sequence and assessing the similarity of that model in three-dimensional space to solved structures of known proteins, for example, stored in curated repositories, such as the Protein Databank (Berman et al., 2000). Many programs are available for protein structural modelling and inference of function. These programs rely on two major approaches including (1) ab initio prediction based on amino acid sequence [e.g., ROBETTA (Kim et al., 2004) and EVfold (Sheridan et al., 2015)] and (2) a guided (Bayesian) approach threading the protein sequence across known solved structures based on underlying sequence similarity [e.g., SPARKS-X (Yang et al., 2011)] and considering the amino acid sequence. Programs such as Swiss Model (Waterhouse et al., 2018), LOMETS (Wu and Zhang, 2007) and MODELLER (Eswar et al., 2007) support inference of protein homology based on comparisons between the predicted protein structure and solved structures for known proteins in curated databases. Software packages including HHpred (Söding et al., 2005), RaptorX (Källberg et al., 2012) and Phyre2 (Kelley et al., 2015) combine threading and homology-based inference in one package. Finally, complex suites of prediction methods, most notably packaged together in the I-Tasser suite (Iterative-threading assembly refinement: Roy et al., 2010), combine ab initio prediction with iterative refinement through guided threading methods, followed finally by homology based inference and predicted

functional annotation. Protein models generated from these predictions can be rendered and visualised in a variety of software packages, including Jmol (Hanson, 2010) and Chimera (Yang et al., 2012).

Accuracy and computational time varies dramatically among these approaches, with complex methods, not surprisingly, being the most computationally intensive and robust. The Critical Assessment of Methods of Protein Structural Prediction (CASP: Moult, 2005) provides an independent benchmarking of these methods, in which programmers are given 24 months (the time between CASP rounds) to provide their best prediction of the structure of ~50–100 unsolved proteins while structural biologists attempt to solve the structure using empirical approaches (e.g., X-RAY crystallography, NMR and Cryo-EM). Programs are scored on a variety of criteria, including the accuracy of the protein backbone, of comparative alignments among the predicted models and similar solved structures, and of ab initio modelling of protein subdomains present in the query protein and absent from related solved structures. Since 2006, I-Tasser has been rated the, or among the, best performing packages for protein structural homology prediction (Roy et al., 2010). The package is limited to proteins under 1500 amino acids and typically performs best for proteins under 750 amino acids. Larger proteins (up to ~3500 amino acids) can be modelled using Phyre2 (Kelley et al., 2015), but only for components of the protein with close homology to known structures (i.e., no ab initio folding).

In an effort to expand on the functional annotation of *G. intestinalis* WB genes, Ansell et al. (2019) undertook genome-wide protein structural homology prediction for most conserved and hypothetical proteins encoded in the *G. intestinalis* WB reference genome, as well as a subset of several hundred proteins with an existing function annotation. These predictions and their underlying meta-data are available for individual or bulk download through http://www.predictein.org/giardia_intestinalis or accessible via individual gene-pages in GiardiaDB. Structural prediction of already functionally annotated proteins can assist in testing and refining these annotations, which for *Giardia* and other non-model organisms are often based on sequence homology only. These predictions can help in identifying ligand or co-factor binding sites; I-Tasser has an in-built function to predict ligand interactions (Roy et al., 2010), or support subsequent in silico docking experiments (Śledź and Caflisch, 2018), for prospective chemical inhibitor development.

Structural homology programs, including I-Tasser, produce a number of quality metrics that can be used to assist researchers in interpreting the outcome and separating higher and lower confidence predictions (Roy et al., 2010). Root mean standard deviation (RMSD) is a common metric, measured in angstroms, to assess the physical distance between the predicted 3D structure of the query protein and its structural homologue among solved proteins in the PDB. This metric is calculated by measuring the distance between each amino acid in the query protein and its nearest structurally equivalent amino acid in the PDB homologue and then computing the overall variance among these distances across the entire predicted model. In general terms, the lower the RMSD value, the more structurally similar are the two proteins in 3D space. In our experience, an RMSD ~<5Å is generally usable for functional inference and, according to Roy et al. (2010), an RMSD <2–3 is of sufficiently

resolved to support subsequent in silico docking and protein-protein interaction modelling. However, this will vary among predictions.

It is important to note that RMSD can only be calculated for the portion of the query model for which a structural homologue is available among the solved PDB proteins. Due to the challenges associated with expressing and crystallising proteins for empirical structural analysis, it is a common occurrence for PDB models to represent only a critical subdomain of a given protein. It is also common for I-Tasser to identify a conserved structural fold in an otherwise diverged protein. It is important to consider the proportion of the query and target models that overlap in the structural homology prediction (calculated as “cov” or coverage by I-Tasser) when inferring a functional annotation from these data. The ab initio software incorporated in the I-Tasser suite is still able to predict a putative structure for the non-overlapping regions of the query protein, but of course these will not contribute to the structural homology prediction. In addition to simple metrics to assess the similarity of the query to a structural homologue, these software packages also produce several composite “confidence” scores. In the case of I-Tasser, this includes a TM-score metric (computed by TM-align), which provides a confidence value based on the RMSD, amino acid similarity and coverage and other metrics, producing a normalized value between 0 (low) and 1. Per Roy et al. (2010), a TM-score about 0.5 is considered to represent a level of structural similarity between the predicted protein and its nearest solved structural homologue that is beyond chance (i.e., biologically informative).

The specific metrics provided by I-Tasser are useful for assessing the quality and reliability of the predicted structure, as well as the functional information that can be derived from its nearest structural homologue. Although Roy et al. (2010) provide advice on quality thresholds for these metrics based on extensive modelling of human proteins, parasites are often highly diverged from model organisms and particularly humans, especially for the deep-branching *G. intestinalis*, and the suitability of human-benchmarked quality thresholds is unclear. Using a machine learning algorithm (Random Forest Classifier), Ansell et al. (2019) sought to develop an organism in-dependent method to assign a confidence score to protein structural homology predictions. In this approach, Ansell et al. (2019) selected *Giardia* proteins with identifiable conserved protein domains based on Interproscan analysis. The Random Forest Classifier was provided each I-Tasser output metric (e.g., RMSD, TM-Score, % amino acid similarity and % coverage) and assessed these for their agreement with the domain comparisons, producing a weighted score that could split into “high” (those query and target proteins that had good domain agreement) and “low” (those that did not) confidence predictions. The Random Forest Classifier (RFC) then applied this weighted scoring system to *Giardia* proteins for which no *a priori* protein domain data were identifiable, separating these predictions in “high-confidence like” and “low-confidence like” categories.

Using the RFC approach, high-confidence functional annotations were assigned to hundreds of hypothetical proteins for which no function data had previously be available (Ansell et al., 2019). Some of these novel annotations include what appear to be key players in critical redox stress systems within the parasite (Ansell et al., 2019), including a novel ortholog of *Entamoeba histolytica* NADPH-dependent oxidoreductase 1, which in the latter species is

implicated in metronidazole activation (Jeelani et al., 2010). Others include putative, novel epigenetic modifiers, that may be important in post-transcriptional or post-translation regulation (Fig. 1). Importantly, I-Tasser functions based on the knowledge that proteins with similar 3D structure tend to have similar functions. However, "function" can include a protein's molecular (i.e., as a kinase, transporter or structural protein) or biological function (e.g., involvement in a specific metabolic or signalling pathway). I-Tasser assigns Gene Ontology classification data based on shared molecular and biological functions of the nearest structural homologues for each prediction. However, in our experience, predicting biological function from structural data alone is unreliable and should be undertaken with caution. As we discuss below, supplementing structural homology prediction data with additional evidence of biological function (e.g., using transcriptomic, proteomic or protein localization data) is advisable.

In silico prediction can be used also to classify and infer functions for non-coding space. RepeatModeller (Tarailo-Graovac and Chen, 2009) and Tandem Repeats Finder (Benson, 1999) are widely used to identify transposable elements and other repetitive sequences. Programs such miRanda (Enright et al., 2003) and PITA (Kertesz et al., 2007) can predict the coding genes regulated by a specific miRNA. PromoterInspector (Scherf et al., 2000) and TRED (Zhao et al., 2005) can model promoter regions and sequence motif modellers, such as DREME (Bailey, 2011), can be used to identify potential transcription factor binding sites. However, most of these in silico predictive methods, aside from repeat finding, have high false discovery rates and should be used with caution or supplemented with additional data. Largely, researchers interested in non-coding regulatory features of the *Giardia* genome are likely better served by "omics" applications, which are discussed below.

4.2 Applying systems biology to function studies

In silico methods provide substantial guidance for researchers interested in *Giardia* molecular biology, but are a starting point to guide empirical experiments. This empirical work includes target-based (e.g., through gene silencing or knockout, or protein over-expression and localization) and systems-based (e.g., through transcriptomic, proteomic or other, similar and broad-spectrum methods) interrogation. This section will focus on systems-based functional study.

To date, transcriptomics and proteomics are the most broadly accessed systems-based approaches for *Giardia*. Franzén et al. (2013) provided the first RNAseq data for *G. intestinalis*, including the only available data to date for an assemblage B (GS) or E (P15) isolate. This work indicated significant sense and antisense transcription in *Giardia* and the authors postulated that *Giardia*'s transcription may be quite "leaky". This observation, coupled with the few transcription factors identified in the genome, suggests *Giardia* exerts little transcriptional level. Despite this, *G. intestinalis* mounts specific transcriptional responses to external stimuli, including ER and starvation stress (Spycher et al., 2013), heat, oxidative-stress and sublethal metronidazole exposure (Ansell et al., 2016) and NO-induced, oxidative-stress and other interactions with host-cells (Ma'ayeh et al., 2015, 2017, 2018).

Not only do transcriptomic data indicate *Giardia* is capable of mounting stress-specific responses (Spycher et al., 2013) and differentiating physiological versus xenobiotic sources

of these stress stimuli (Ansell et al., 2016), transcription responses differ among *Giardia* isolates. A recent study (Ansell et al., 2017) followed the transcriptional changes of *G. intestinalis* WB, 713 and 106, preceding and upon development of metronidazole resistance, identifying dynamic balancing between oxidative stress responses and metabolic activity, but also finding major differences among isolates. Metronidazole resistance in *G. intestinalis* WB and 713 appears to centre around up-regulation of oxidative stress response coupled with downregulation of the glycolytic system and reliance on arginine metabolism. This shift seems to come at a substantial fitness cost that manifests, particularly in *G. intestinalis* WB, as a reduced in vitro growth rate. In contrast, the transcriptional response to metronidazole resistance by *G. intestinalis* 106 appears focused on changes in nitroreductase activity, including a shift from metronidazole activating, nitroreductase-1 (NR1) transcription, to metronidazole deactivating, nitroreductase-2 (NR2) transcription. Proteomic data for these isolates show that transcriptional changes lead to dynamic responses at the protein level (Emery et al., 2018). Emery et al. (2018) also found that proteomic changes associated with *G. intestinalis* 106 were stable after 24 weeks of passage in in vitro in the absence of metronidazole. In contrast, most likely owing to an increased fitness cost, metronidazole resistance rapidly decayed of the same in vitro selection experiments when either *G. intestinalis* WB or *G. intestinalis* 713 isolate was tested, as has been described for these isolates elsewhere (Ansell et al., 2015b).

Transcriptomic and proteomic datasets supports *Giardia*'s coding gene models and improves mapping of gene boundaries, UTRs, splice junctions and other regulatory features. Numerous "omics" based approaches are available to expand on this knowledge, refining our understanding of the overall genomic and epigenetic regulation in *Giardia*, and supporting identification of novel players in important regulatory, signalling, stress response, metabolic and myriad other essential cellular functions. Many of these methods are available, but yet to be applied to *Giardia*. For example, further studies employing the targeted immunoprecipitation of AGO and other RNA-interacting proteins (e.g., per DICER: Saraiya et al., 2014) could greatly improve understanding of the importance of small RNAs in *Giardia* biology and identify their specific target genes. Chromatin-immunoprecipitation (ChIP) sequencing (Park, 2009) can be used to better understand the role of specific histone modifications in chromatin-level regulation in the cell cycle, during encystation/excystation and antigen switching, and following drug exposure or in vitro selected resistance.

Clearly, there are a variety of circumstances in which transcriptomic and proteomic approaches can be further employed to dissect parasite behaviour. Similarly, although several studies have indicated an important role for protein methylation, phosphorylation and acetylation (e.g., in encystation or drug resistance; reviewed in Ansell et al., 2015b), there are as yet no broad-scale studies of these modifications in *Giardia*. Metabolomics presents a considerable and as yet almost entirely untapped method to explore functional biology of *Giardia*, and would further open up more quantitative proteomic technologies (e.g., SILAC). However, metabolomics research is most powerful in its application in quantifying the rate of change (i.e., flux) in metabolites over time. This requires a defined media that can be altered to include radioactively labelled carbon, nitrogen or phosphorous, which is currently not available for *Giardia*.

As “omics” resources are developed for *Giardia*, it becomes possible to leverage their combined value in integrative analyses either combining datasets of the same (e.g., transcriptomics) or multiple origins (e.g., transcriptomics, proteomics and metabolomics data). R-language scripts, such as voom (Law et al., 2014), support identification and removal of systematic biases or batch effects between studies (this is essential for accurate analysis). Methods such as weighted-gene co-expression network analysis (WGCNA: Langfelder and Horvath, 2008) support identification of genes that strongly correlate in their transcriptional or expressional behaviour across a variety of complex conditions (e.g., stage differentiation, stress responses, cell division, etc.). Such tightly correlated genes are often co-regulated and tend to have related biological functions. These approaches can be used to infer novel functions for genes even with no *a priori* knowledge of their biology, for example, based on their co-regulation with multiple well annotated genes.

In-direct methods provide powerful, systems-level tools to explore the molecular biology of non-model organisms, such as *Giardia intestinalis*. They are, of course, a starting point for detailed functional research and can greatly assist development of specific, direct experiments for further exploration. Advances in tools for direct empirical study of gene function in *Giardia* are discussed in the next section.

5. Direct, targeted-based assessment of gene function

As most parasites are unculturable and genetically intractable, much of the functional biology is understood through in-direct inference by in silico modelling and, more recently, “omics” and systems-based research. These are powerful tools, but are most valuable when they are used to guide empirical interrogation of gene function through direct, target-based studies. For many years, *Giardia* was proposed as a model parasite because of its ready in vitro cultivation, but significant challenges in its genetic manipulation confounded this potential and stymied critical efforts to better understand the parasite. Recent major advances in the application of functional genetic tools to *Giardia* will open up exciting pathways for research in the coming years. These advances are discussed here.

5.1 Transfection vectors in *Giardia* and their use in studies of *Giardia* biology and pathogenesis

The first report of transient DNA transfection of *Giardia* was published in 1995 by Yee and Nash (1995). The plasmids were of bacterial origin and based on the pGEM (Promega) backbone containing the luciferase (*luc*) gene driven by 82bp upstream sequence of the glutamate dehydrogenase (*gdh*) gene, the first 18 *gdh* codons fused to *luc* and 129bp of downstream sequence of *gdh*, including the polyadenylation signal (Yee and Nash, 1995). Expression of luciferase required 50 μg of pure plasmid DNA in the transfection, and optimal expression was seen after 6h, decreasing to 13% after 24h (Yee and Nash, 1995). This system was further developed to a stable transfection system by insertion of a *gdh* regulated puromycin-*N*-acetyltransferase gene (*pac*) in to the transient transfection vector (Singer et al., 1998). Stable transfectants could be obtained after selection in 100μM puromycin (current standard concentrations for stable transfection of *Giardia* using *pac* containing plasmids is 50 μg/ml) and the plasmids were shown to be episomal (Singer et al.,

1998). Later studies showed that the plasmid localized randomly to one of the two *Giardia* nuclei for up to 1 year during selection in puromycin (Yu et al., 2002). An integration vector was constructed using fragments of the triose phosphate isomerase (*tpi*) gene and integration was shown to be more effective in the assemblage B isolate GS than in the assemblage A isolate WB (Singer et al., 1998). This study also showed that the green fluorescent protein can be used as a reporter gene in *Giardia* (Singer et al., 1998). Another study showing stable transfection of *Giardia* using bacterial neomycin phosphotransferase (*neo*) as selectable marker in the pGEM plasmid was published by Sun et al. (1998) at the same time. The neo gene was driven by regulatory fragments from the giardial RAN gene and 150 μg/mL G418 was used in the selection. An increase of the amount of neomycin in the medium from 150 to 1200 μg/mL G418 increased the copy number of the plasmid, and it was shown that the plasmid remained in the cells for up to 50 days without selection (Sun et al., 1998).

The replication of external DNA fragments without selection has been seen in several later publications (Ebneter et al., 2016; McInally et al., 2019). The *pac-gdh* cassette was transferred to the pBlueScriptII KS(+)(Stratagene) vector and a deletion study was performed on the encystation-specific promoter of glucosamine-6-phosphate isomerase (Gln6PI-B) using luciferase as reporter (Knodler et al., 1999). This showed that only 50bp upstream of the start codon of Gln6PI-B is needed for developmental regulation, with this region later shown to bind the encystation-specific transcription factor Myb2 (Sun et al., 2002). The RAN-neo cassette was moved into the pBlueBcript vector by Hehl et al. and they could show that cyst wall protein 1 (CWP1) is regulated by a 110bp upstream sequence (Hehl et al., 2000). Importantly, they also showed, by using CWP1-GFP fusions, the mechanism by which cyst-wall material is transported in encystation-specific vesicles (ESVs) out to the emerging cyst-wall in encysting cells (Hehl et al., 2000). Thus, gene regulatory fragments and protein localization signals can be studied in *Giardia* since year 2000. Other inducible systems in *Giardia* including use of promoters of CWP-1 and −2 have later been used (Ebneter et al., 2016) and a tetracycline controlled gene expression system (Sun and Tai, 2000). Nonetheless, there is a need for new inducible gene expression systems in *Giardia* since both the tetracycline system is not very tightly controlled, and the CWP-system dependent on encystation induction. A new system for rapid triple-hemagglutinin (3HA) tagging and integration of *Giardia* genes into their endogenous regions was developed by Gourguechon and Cande (2011) and it was shown that blasticidin (75 μg/mL) can be used as a third selectable marker in *Giardia* by using a blasticidin resistance gene from the plasmid pBOSH2BGFP (Clontech).

The need to over-express and purify specific *Giardia* proteins for further characterization in vitro, as well as purify protein complexes, sparked the development of a suite of cassette-based expression vectors (Jerlstrom-Hultqvist et al., 2012). These vectors contain N- and C-terminal streptavidin binding peptide-glutathione S-transferase (SBP-GST) tags for production of recombinant proteins and N- and C-terminal Strep II-FLAG-tandem affinity tags for tandem affinity (TAP) purification of protein complexes (Jerlstrom-Hultqvist et al., 2012). The *Giardia* virulence factor arginine deiminase (ADI) and the proteasome complex were purified as proofs of concept. This suite of vectors has multiple cloning sites for cloning of genes of interest and have been modified to contain 3HA, 6 × His and the tetracycline regulatory system (Einarsson et al., 2016a).

In addition to over-expression, targeted gene repression or knockout is a major step in understanding protein function. However, *Giardia* is a tetraploid organism with two diploid nuclei (Bernander et al., 2001; Yu et al., 2002). This makes it difficult to genetically deplete proteins for functional analyses. The development of molecular genetic tools for gene knockout in *Giardia* has lagged behind that of other parasitic protists (DiCarlo et al., 2013; Hwang et al., 2013; Mali et al., 2013; Wang et al., 2013) or other polyploid organisms (Bedell et al., 2012; Clark et al., 2011). A rudimentary understanding of transcription factors and promoters (Davis-Hayman and Nash, 2002) and a limited number of selectable markers (Bedell et al., 2012; Clark et al., 2011; Gourguechon and Cande, 2011) have contributed to the slow pace of tool development. The giardial Cre/*loxP* system was used by Ebneter et al. (2016) to generate the first true knock-out mutants of *Giardia* lacking one to four copies of the CWP-1 gene. Knocking out one or two of the four copies did not create any detectable phenotypes, but trophozoites with one or no CWP-1 gene copy did not produce viable cysts and had impaired transport of CWP-2 in ESVs (Ebneter et al., 2016). Disc and flagellar disassembly and nuclear division still occurred, generating pseudocysts (Ebneter et al., 2016). The generation of the first knock-out mutants was major step in *Giardia* research, but this approach is onerous in that it has to be done sequentially with recycling of selectable markers, and it takes a long time to generate parasites with all four alleles knocked-out. Strategies beyond gene knockout are critical when assessing the functions of unknown proteins (Qi et al., 2013). Knockouts of genes in essential metabolic pathways or processes such as cell division can be lethal (Hardin et al., 2017), and require alternative strategies such transcriptional repression or translational blocking are also useful tools for determining protein function.

The *Giardia* genome contains conserved components of the RNA interference (RNAi) machinery, yet RNAi, a powerful tool for gene silencing in many organisms, is inefficient in *Giardia* (Krtkova and Paredez, 2017; Marcial-Quino et al., 2017). Other transcriptional repression methods, such as the overexpression of long double-stranded RNAs or hammerhead ribozymes (Chen et al., 2007; Dan et al., 2000), also have been used in *Giardia* with limited success and reproducibility. Morpholino oligonucleotides, which act by blocking translation, have been used most extensively for gene knockdown (Dawson et al., 2007; Hoeng et al., 2008; House et al., 2011; Paredez et al., 2011; Woessner and Dawson, 2012). However, although morpholino knockdowns can be robust in *Giardia*, morpholinos are expensive, and are thus less applicable for genome-wide functional screens (Krtkova and Paredez, 2017). Morpholinos can also lack complete penetrance and are transient, lasting less than 48h after electroporation. These limitations make them less than optimal for the characterization of infection dynamics in animal hosts (see below and Barash et al., 2017).

5.2 Creating robust and stable gene knockdown strains with CRISPR interference

CRISPR/Cas9 gene editing provides a potentially transformative technology for *Giardia* research. However, the use of CRISPR/Cas9 in *Giardia* was stalled by the parasites lack of a non-homologous end-joining (NHEJ) pathway (Morrison et al., 2007) and a defined sexual cycle (Poxleitner et al., 2008), as well as problems with nuclear localization of Cas9 using standard nuclear localization signals (SV40 NLS: Ebneter et al., 2016). Nonetheless, CRISPR/Cas technology has revolutionized genome editing in eukaryotes, and CRISPR/

Cas-mediated knockout strategies have recently been implemented for several parasitic protists (Ren and Gupta, 2017). Beyond their utility for gene knockout, Cas proteins have been exploited for their ability to bind to target nucleic acid sequences and recruit a variety of effector proteins, including transcriptional repressors and activators, epigenetic modifiers and fluorophores for imaging (Larson et al., 2013; Pickar-Oliver and Gersbach, 2019). In both eukaryotic and bacterial model systems, CRISPR interference (CRISPRi) has been shown to be a robust alternative to RNAi-mediated gene silencing for knockdown of gene expression (Kampmann, 2018; Larson et al., 2013).

CRISPRi is a modification of the CRISPR/Cas system using a catalytically inactive, or "dead", Cas protein (dCas) to promote stable, inducible, or reversible gene knockdown in human cell lines and yeast (Larson et al., 2013) and diverse bacteria (Kaczmarzyk et al., 2018; Larson et al., 2013; Liu et al., 2017; Tao et al., 2017; Zhang et al., 2016; Zuberi et al., 2017). Like Cas proteins, inactive dCas proteins are directed to precise genomic targets using a complementary guide RNA (gRNA). Rather than catalysing double-stranded breaks in DNA, the inactive dCas/gRNA complex prevents transcription initiation and/or elongation upon binding (Larson et al., 2013). CRISPRi is as effective as RNAi in transcriptional silencing, and fewer off-target effects have been reported (Larson et al., 2013). Thus CRISPRi directly and stably inhibits transcription, and offers significant advantages over RNAi or morpholino knockdown (Larson et al., 2013). CRISPRi does not interfere with endogenous microRNA expression or function (Larson et al., 2013), and because CRISPRi acts at the DNA level, *Giardia* noncoding RNAs (Saraiya and Wang, 2008), microRNAs (Li et al., 2011; Zhang et al., 2009), antisense transcripts (Elmendorf et al., 2001), and polymerase III transcripts (Hudson et al., 2012) could be targeted for repression.

CRISPRi was recently adapted to repress both exogenous and endogenous genes (McInally et al., 2019) in *Giardia*. CRISPRi does not require *Giardia* host or viral factors as is required for antisense (Rivero et al., 2010b) or hammerhead ribozyme-mediated transcriptional repression (Chen et al., 2007; Dan et al., 2000). In *Giardia*, targeting of the *Streptococcus pyogenes* Cas9 or dCas9/gRNA DNA recognition complex to both nuclei required the addition of a native *Giardia* nuclear localizing sequence (NLS) (McInally et al., 2019). A CRISPRi episomal vector (dCas9g1pac) was then created that includes cassettes for the expression of dCas9 and a gRNA, as well as a marker for puromycin resistance for positive selection in *Giardia* (Fig. 2). *Giardia* gRNAs are designed using the CRISPR Guide RNA design tool at Benchling [Biology Software] (Benchling, 2019) or the Eukaryotic Pathogen CRISPR guide RNA/DNA Design Tool (EuPaGDT) (Peng and Tarleton, 2015) at GiardiaDB (giardiadb.org), and are chosen based on predictions of gRNA efficiency and the results of on- and off-target analyses. Complementary gRNA oligonucleotides are then annealed and cloned into the CRISPRi vector using a one-step digestion/ligation reaction. gRNA design and cloning can be accomplished in a week, and CRISPRi strains are obtained 10–14 days after electroporation of constructs into *Giardia* trophozoites.

Stable, precise and robust CRISPRi-based gene regulation of single or multiple genes will transform the study of molecular and cellular biology and pathogenesis in this widespread intestinal parasite. The modular design of the *Giardia* gRNA expression cassette allows for the concatenation of more than one gRNA to target multiple sites in one target gene, or more

than one *Giardia* gene or gene family. Once imported into the nuclei, the dCas9/gRNA complex is targeted to a specific genomic locus where it sterically interferes with RNA polymerase or transcription factor binding or with transcriptional elongation, as has been shown in bacteria or other eukaryotes (Larson et al., 2013). dCas9 expression and overall penetrance is quantified through the use of anti-Cas9 antibodies to score dCas9-positive trophozoites. The use of FACS and other methods to enrich for trophozoites that strongly express dCas9 could increase knockdown penetrance, as has been seen in other CRISPRi systems (Gilbert et al., 2013).

In every CRISPRi system (Larson et al., 2013), the choice of gRNA target site has been shown to impact the degree of transcriptional repression. In contrast to human cells (Gilbert et al., 2013; Qi et al., 2013), targeting gRNAs close to the transcriptional start site (TSS) results in stronger repression in some bacterial systems. To determine how gRNA positioning influenced the magnitude of transcriptional repression in *Giardia*, 11 different gRNAs were systematically designed to target the transcriptional start site and the coding region of the bioluminescent NanoLuc (NLuc) gene in 50-bp increments (McInally et al., 2019). There was no correlation between gRNA position and the magnitude of repression; however, in general, more repression was observed with gRNAs that targeted the first 200bp of the coding region. Because gRNAs targeted the coding region rather than the promoter sequence used for NLuc expression, it is likely that efficient transcriptional repression occurred via the inhibition of transcriptional elongation, rather than inhibition of transcriptional initiation (Larson et al., 2013). For successful gene repression in *Giardia*, several gRNAs should be tested for each target, as has been suggested for CRISPRi in human cells (Gilbert et al., 2013). The ability to direct dCas9 to both nuclei in *Giardia* could enable the modulation of transcriptional networks not only by repression, but also by differential expression or overexpression through the fusion of dCas9 to *Giardia*-specific transcription factors, enhancers, or other native transcriptional elements (Kampmann, 2018).

Giardia's dynamic microtubule cytoskeleton is of critical importance throughout both stages of its life cycle (Dawson, 2010; Nosala and Dawson, 2015). CRISPRi-mediated stable transcriptional repression was also used recently to knock down the expression of three endogenous cytoskeletal proteins, highlighting the versatility of CRISPRi to interrogate *Giardia* microtubule functioning in flagellar motility and disc-mediated attachment (McInally et al., 2019). Specifically, 2 of the 24 *Giardia* kinesin motor proteins (kinesin-2a and kinesin-13) and one ventral disc protein (DAP16343) were targeted for CRISPRi knockdown. The heterotrimeric kinesin-2 motor is required for assembly of external regions of axonemes (Dawson et al., 2007; Hoeng et al., 2008). Kinesin-13 regulates flagellar length in all *Giardia* axonemes through the promotion of microtubule disassembly at the distal flagellar tips (Dawson et al., 2007). Overexpression of dominant negative kinesin-2 or kinesin-13 thus results in aberrant flagellar lengths of all of the eight flagella (Dawson et al., 2007; Hoeng et al., 2008). Similar length defects were observed with the CRISPRi-based knockdowns of kinesin-2a and kinesin-13, supporting the essential roles of these two kinesins in flagellar length regulation in *Giardia*.

Over 90 proteins compose *Giardia*'s ventral disc. Prior transient morpholino-based knockdown of the disc-associated protein DAP16343 (median body protein, or MBP)

resulted in discs with an open and flattened conformation and significant attachment defects (Woessner and Dawson, 2012). Stable CRISPRi-mediated knockdown of MBP resulted in the same “open” and “flat” disc phenotype observed with morpholinos (Woessner and Dawson, 2012), again confirming the use of CRISPRi as a genetic tool to affect disc structure and attachment. In contrast to morpholino-based knockdowns, the MBP knockdown strain is stable over time and phenotypes are 60–100% penetrant in Cas9+ cells. As noted previously, the degree of CRISPRi knockdown of cytoskeletal genes was contingent upon the targeting position of the gRNA and the degree of expression of dCas9 in the nuclei. More severe phenotypes were associated with dCas9 positive cells, with overall population-level transcriptional knockdowns ranging from 10 to 60%. Rather than a deficit, the variations in knockdown levels highlight the overall tunability of this system for knockdown of endogenous genes in contrast to transient morpholino knockdown. The ability to examine cells with varying degrees of knockdown and to reduce the severity of phenotypes by selecting gRNAs with less than complete transcriptional repression are critical toward the evaluation of essential *Giardia* genes. Furthermore, this work demonstrated the ability to knock down two different genes simultaneously with the concatenation of gRNAs targeting both kinesin-13 and MBP. This is a key technology for evaluation of the redundant functions of genes in a gene family or pathway.

5.3 Using CRISPRi to study basic and pathogenic aspects of *Giardia* biology

CRISPRi will rapidly change how we study basic cell biology, development, and pathogenesis of *Giardia*. CRISPRi would be an inexpensive and fruitful strategy to identify essential genes associated with key aspects of *Giardia’s* life cycle, such as attachment (Nosala et al., 2018), motility (Dawson and House, 2010), or encystation (Einarsson et al., 2016c; Pham et al., 2017). The ability to rapidly create stable knockdown strains will facilitate the functional identification of genes involved in pathogenicity (e.g., motility or attachment) that can then be screened to identify druggable targets. Genomes and genetic tools are available for the assemblage A strain WBC6 (Morrison et al., 2007) and the assemblage B strain GS (Davis-Hayman and Nash, 2002); however, no genetic tools have been developed for other sequenced assemblage A strains such as DH (Adam et al., 2013), or for other human clinical A and B assemblage isolates (Hanevik et al., 2015). Thus, ongoing work should also aim to develop genetic tools in other *Giardia* strains of clinical relevance.

5.4 In vivo bioluminescent imaging of *Giardia* infection dynamics in small animal models

The molecular and cellular mechanisms underlying *Giardia’s* colonization and differentiation into cysts in the gastrointestinal tract remain unclear. Mammalian hosts ingest *Giardia* cysts that excyst and release motile trophozoites as they transit into the gastrointestinal tract (Einarsson et al., 2016b). The excysted trophozoites then attach and extracellularly colonize the epithelium of the small intestine. Later, trophozoites detach and encyst and these cysts are disseminated in faeces (Adam, 2001). Most studies of *Giardia’s* pathogenesis have relied on in vitro models of giardiasis that may not be adequate proxies for infection within the host or may not accurately reflect in vivo parasite physiology. In vivo studies of giardiasis have been limited by the inaccessibility of the intestinal tract; thus, indirect quantification methods have been used to assess in vivo parasite burden,

differentiation, and physiology, including the isolation of cysts from faeces or the isolation and enumeration of parasites from the intestines of euthanized study animals (Bartelt et al., 2013; Solaymani-Mohammadi and Singer, 2011). The inaccessibility of the site of *Giardia* colonization in the gut has restricted tracking temporal infection dynamics in a single animal throughout the course of infection, and instead necessitated the use of cohorts of animals for each experimental time point for adequate experimental rigour and reproducibility (Barash et al., 2017). Furthermore, *Giardia* colonization of the small intestine overlaps with ecological niches inhabited by commensal microbiota, yet in vivo *Giardia*-microbiome interactions have largely been ignored in models of pathogenesis (Bartelt and Sartor, 2015).

The adhesive disc of *Giardia* is a unique cytoskeletal structure containing more than 100 proteins (Hagen et al., 2011). In order to study putative adhesive disc proteins, identified by sub-cellular fractionation and proteomic analyses, the Dawson lab developed a ligation-independent high-throughput cloning method (Gateway cloning) for C-terminal GFP or mNeon green (NG) tagging of *Giardia* proteins (Hagen et al., 2011). This system has been used to determine the subcellular localization of selected *Giardia* proteins that lack homology or have limited similarity to proteins in other organisms (www.Giardiadb.org). The ORFs plus 200bp of upstream sequence containing the native promoter were PCR-amplified from genomic *Giardia* DNA to create Gateway entry clones and subsequently cloned into the Gateway expression vector pcGFP1F.pac (Hagen et al., 2011) to create C-terminal GFP fusion strains. Until now ~600 proteins have been localized in *Giardia* using this system and the data is available at *Giardia* DB.

Recently developed methods for bioluminescent imaging (BLI) using integrated luciferase bioreporter strains now allow real-time quantitative visualization of the temporal and spatial dynamics of *Giardia* infections in the gastrointestinal tracts of small animal (Barash et al., 2017). BLI has been used previously to monitor parasitic infection dynamics for malaria, leishmaniasis, trypanosomiasis, and toxoplasmosis (D'Archivio et al., 2013; Reimao et al., 2013; Saeij et al., 2005), as well as bacterial colonization of the intestine (Hutchens and Luker, 2007). In general, BLI enables sensitive quantification and real-time reporting of metabolic activity via imaging of the transcriptional activity of promoter-luciferase fusions or imaging of protein expression through the expression of native proteins fused to luminescent proteins such as NanoLuc (Luo et al., 2006; Weissleder and Ntziachristos, 2003; Welsh and Kay, 2005). Non-invasive in vivo BLI relies on the external detection of light produced internally, and signal intensity may be limited by the overall level of luciferase expression, the oxygen tension within relevant tissues, pigmentation of organs and skin, or any background signal from the animal (Andreu et al., 2011). However, the gut is sufficiently oxygenated to permit signal detection. Importantly, although animal tissues exhibit relatively high background levels of autofluorescence, they have nearly nonexistent levels of autoluminescence, which facilitates bioluminescent signal detection even at low signal strength (Andreu et al., 2011; Foucault et al., 2010; Rhee et al., 2011).

In vivo models of infection are needed to help define the complex interactions between *Giardia* and the gastrointestinal tract of the mammalian host. Zoonotic *Giardia* strains have varied physiologies and assemblage A (strains WBC6 and DH) and assemblage B (strains GS and HS) (Ankarklev et al., 2015; Sprong et al., 2009) are the only assemblages identified

in human infections. Animal models of giardiasis have used adult (Byrd et al., 1994; Singer, 2016) or suckling mice (Mayrhofer et al., 1992) or adult gerbils (Rivero et al., 2010a) infected with either human isolates of *Giardia* assemblages A (strain WBC6) or B (strains GS or H3), or murine *G. muris* isolates (Aggarwal and Nash, 1987). Assemblage B strain H3 cysts have been used to establish animal infections (Bartelt et al., 2013), yet strain H3 currently lacks a genome sequence, and cannot be transfected or otherwise genetically manipulated. Variation in the degree or timing of experimental infections have been noted with the use of isolates from different assemblages or the use of different animal models, and mirrors variability in human giardiasis (Watkins and Eckmann, 2014). Such variation can confound the interpretation of infection dynamics from experimental cohorts and underscores the need for additional methods of evaluating *Giardia* infections in animal models.

In vivo BLI offers both real-time and longitudinal monitoring of infection dynamics in small animals such as mice or gerbils. Infection with recombinant *Giardia* strains containing integrated bioluminescent proteins such as firefly luciferase fused to physiological or encystation-specific promoters or genes (commonly termed “bioreporters”) has permitted the temporal and spatial imaging and quantification of parasite colonization, metabolism and differentiation (Barash et al., 2017). As the WBC6 (assemblage A) strain is genetically tractable, three transcriptional bioreporter strains have been created for in vivo and ex vivo BLI by integrating firefly luciferase driven by either endogenous metabolic or encystation-specific promoters (Barash et al., 2017). Infections with the integrated constitutive glutaraldehyde dehydrogenase (GDH) bioreporter (P_{GDH}-*FLuc*) strain allow the quantification of in vivo infection dynamics including parasite density and metabolism. Optical imaging with this constitutive strain directly correlates with qPCR-based measures of parasite density (Barash et al., 2017). Luciferase expression from the GDH promoter continues at significant levels for at least 24h after the P_{GDH}-*FLuc* strain is transferred to encystation medium; therefore, BLI of constitutive metabolic genes could be used as a proxy for in vivo *Giardia* abundance even during encystation. BLI of infections with the constitutively expressed P_{GDH}-*FLuc* strain confirm that maximal infection occurs at approximately 7 days, consistent with prior studies of experimental *Giardia* infections in mice (Byrd et al., 1994). In vivo *Giardia* physiology and encystation in cohorts of mice or gerbils have been monitored non-invasively for at least 21 days. Longitudinal BLI of the same study animal provided a robust method to estimate variance within infections, which is essential for determining statistically informative study animal numbers. Additional in vivo bioreporter strains could include those associated with parasite stress response, physiology, or excystation. In vivo parasite protein expression could also be monitored if *Giardia* proteins were fused to bioluminescent proteins such as NanoLuc (Stacer et al., 2013), which could be particularly informative for the in vivo quantitation of secreted *Giardia* proteins.

Ex vivo BLI of tagged *Giardia* strains has enabled the spatial quantification of colonization and parasite differentiation in the GI tract of mice and gerbil animal models. After live imaging, study animals are euthanized, and the GI tract is excised and imaged again with BLI to provide greater spatial resolution. Quantitative ex vivo BLI of the constitutive bioreporter P_{GDH}-*FLuc* in both mice and gerbils showed that rather than uniformly colonizing throughout a region of the GI tract, *Giardia* colonizes the proximal small intestine

non-uniformly in discrete high-density foci. Such BLI-based anatomical sampling strategies facilitate the precise identification of regions of the gastrointestinal tract associated with high or low densities of colonizing or encysting parasites. In this way, ex vivo spatial localization of bioreporter signals can be used to correlate discrete areas of *Giardia* abundance and encystation in the gastrointestinal tracts of study animals with spatially defined histological analyses or transcriptional profiling of the same discrete *Giardia* foci (Pham et al., 2017).

Trophozoites are believed to be induced to encyst after receiving biochemical cues from a specific gastrointestinal anatomical site (Gillin et al., 1987; Lujan et al., 1997). Encystation can be induced in vitro by lowering the pH or adjusting the concentrations of other components of the medium, particularly bile (Gillin et al., 1988; Lujan et al., 1996). Parasite commitment to encystation and excystation are key highly regulated transitions in *Giardia*'s life cycle (Einarsson et al., 2016c). Hallmarks of the developmental commitment to encystation include the significant transcriptional upregulation of genes encoding the cyst wall proteins CWP1 and CWP2, and the appearance of encystation specific vesicles (ESVs) that transport the cyst wall proteins (e.g., CWP1 and CWP2) to build the cyst wall (Hehl et al., 2000; Lujan et al., 1995).

To monitor the temporal and spatial dynamics of in vivo encystation with BLI, two integrated encystation-specific bioreporters (P_{CWP1}-*FLuc* and P_{CWP2}-*FLuc*) have also been developed (see Fig. 3). By imaging encystation-specific bioreporters, it was observed that encystation initiates shortly after inoculation and continues throughout the entire duration of infection. Using ex vivo BLI, encystation initiation was reported to occur in discrete foci within the proximal and distal small intestine, rather than uniformly throughout a particular region of the gut. Thus *Giardia* BLI has uncovered that parasites colonize and encyst in the gastrointestinal tract of both mice and gerbils with a localized or "patchy" distribution (Barash et al., 2017), as has been observed for many other pathogens of the gastrointestinal tract or other organs (Contag et al., 1995; D'Archivio et al., 2013; Foucault et al., 2010; Reimao et al., 2013; Saeij et al., 2005). To discriminate between encysting and non-encysting parasites in ex vivo samples, dual reporter bioluminescent *Giardia* strains using two spectrally different bioluminescent tags (such as firefly luciferase and red-shifted firefly luciferase) could be used to simultaneously image and quantify metabolically active ($_{GDH}$-*FLuc*) and encysting ($_{CWP1}$-*RLuc*) parasites (Branchini et al., 2007; Cevenini et al., 2014; Maguire et al., 2013).

Ultimately, the use of single or dual spectra bioreporters of additional metabolic genes will provide a more comprehensive understanding of the in vivo interactions of *Giardia* with the host or with the microbiome. *Giardia* trophozoites produce no known toxin, and colonization does not elicit a robust inflammatory reaction (Bartelt and Sartor, 2015; Cotton et al., 2015). Although it has been speculated that *Giardia* infection results in intestinal epithelial damage, changes in the ultrastructure of the small intestine that are sufficient to cause clinical symptoms have not been consistently observed (Robertson et al., 2010). With BLI-directed ex vivo sampling of foci of colonization, one can precisely correlate trophozoite abundance and metabolism with host pathology in the same anatomical region.

The ability to create stable CRISPRi knockdowns in the luciferase bioreporter strains used for bioluminescent imaging (BLI) will be essential toward understanding the cellular roles of *Giardia* proteins during colonization and encystation in the host (Barash et al., 2017). Combined with BLI, CRISPRi will enable the examination of fitness, colonization, or encystation differences in knockdown *Giardia* strains. BLI has also been validated for the analysis of anti-*Giardia* drugs by the demonstration that metronidazole, the standard of care anti-*Giardia* drug that targets parasite metabolic activity (Tejman-Yarden and Eckmann, 2011), reduced in vivo bioluminescence of the constitutively-expressing P_{GDH}-*FLuc* bioreporter strain. As it has been used extensively to evaluate the efficacy of anti-parasite or anti-bacterial drugs (Xu et al., 2016), BLI will be valuable as an alternative and real-time method to evaluate anti-*Giardia* drugs in relevant animal models of giardiasis.

6. Concluding remarks

Giardia has proven a tantalizingly close but frustratingly distant model eukaryote and genetically tractable parasitic species. Its ready culturability and early discoveries in its regulatory biology, as well as its status as an early diverging branch of the eukaryotic tree and cause of significant disease in humans and animals, has piqued interest in the species for many years. However, its binucleate cell, tetraploid genome and limited acceptance of seemingly attractive genetic tools, such as RNA interference and conventional gene knockout, have stymied research and limited opportunities for researchers to slake their interest. These limitations appear largely over-come and *Giardia* is poised to take its place as a model system that can be studied both as a significant infectious organism and as an early glimpse into evolution of eukaryotic biology, metabolism and regulatory systems. Advances, particularly in the demonstration of CRISPr-based methods for genetic manipulation, will no doubt shape the field for many years to come. Some challenges still remain, particularly in the ability to generate stable gene knockouts and the need for a defined culture media to support label-based proteomic and metabolomic studies. Yet, the primary tools are now in place, the genomic and systems-based resources established, and the limitations to its exploration now appear readily surmountable. The field is poised to enter a new and exciting era of biology discovery, in which we, and no doubt our many colleagues, are eager to take part.

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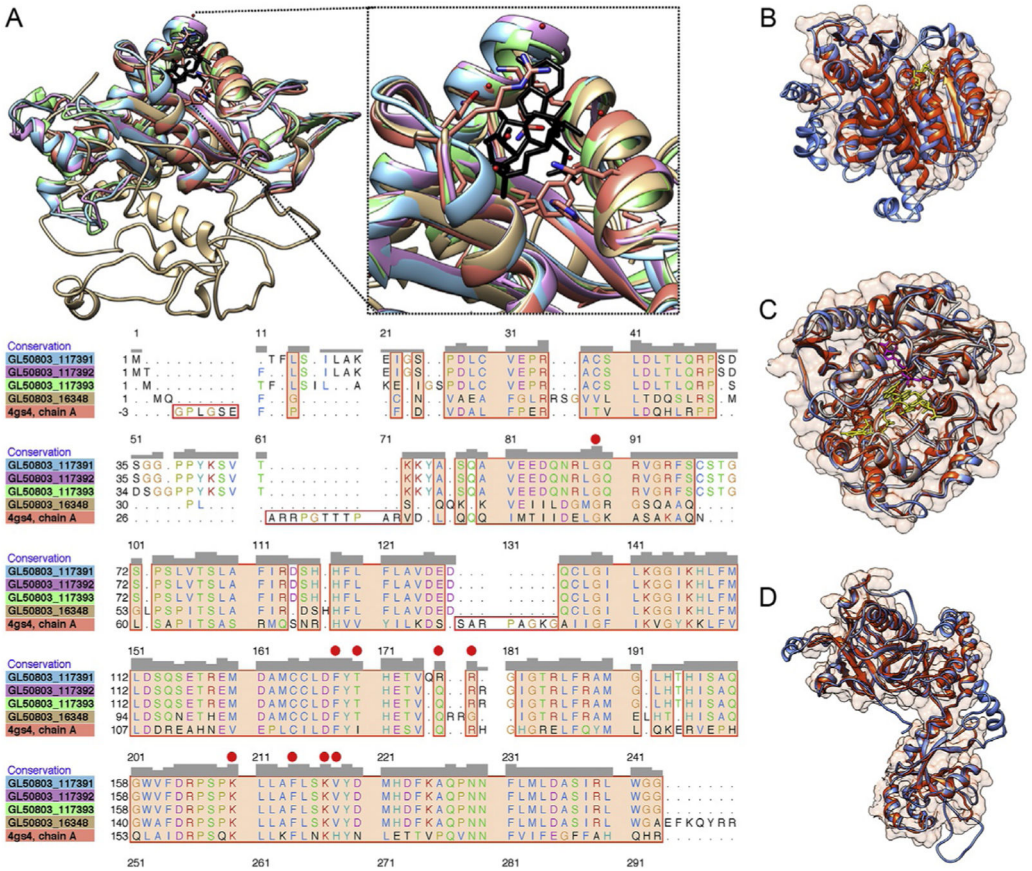

Fig. 1.

Novel functional annotations for *Giardia* proteins based on 3D structural homology. Examples of high-confidence models with potential and novel roles in post-transcriptional and post-translation regulation in *Giardia* include (A) Several homologues of human α-tubulin acetyltransferase 1 (PDB ID: 4GS4; red). Protein model includes overlay of *G. intestinalis* WB C6 (GL50803) coding genes GL50803_16348 (gold), GL50803_117391 (blue), GL50803_117392 (purple) and GL50803_117393 (green). Inset (dotted border) shows expanded view of the α-tubulin binding pocket containing an acetyl-CoA (black). Sequence alignment shows conserved residues among all models. Red circles in the alignment depict core catalytic residues required by human αTAT1 for acetylating α-tubulin. (B) A *Giardia* homologue [GL50803_22338 (blue)] of human mRNA cap guanine N7-methyltransferase [5′ m7g MTase; PDB ID: 3BGV (red)], showing S-Adenysl-L-Homocysteine in the RNA-binding pocket; (C) Two homologues [GL50803_100887 (blue) and GL50803_103058 (grey)] of yeast cap methyltransferase 1 (red), showing S-adenosyl-L-methionine (purple) and 7N-Methyl-8-Hydroguanosine-5′-Triphosphate (yellow) in RNA-binding pocket, and (D) one homologue [GL50803_17308; (blue)] of yeast Cet1 mRNA guanine-N7 capping guanylyltransferase [PDB ID: 3KYH (red)]. *Data from Ansell, B.R.E., Pope, B.J., Georgeson, P., Emery-Corbin, S.J., Jex, A.R., 2019. Annotation of the Giardia proteome through structure-based homology and machine learning. Gigascience 8, giy150.*

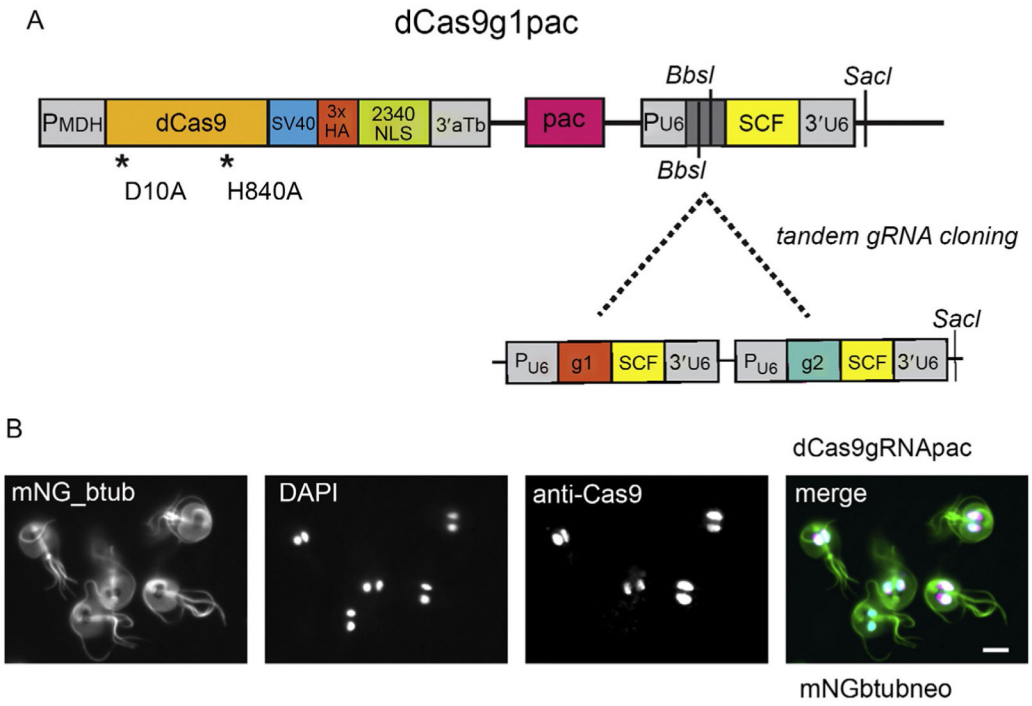

Fig. 2.

The Giardia CRISPRi plasmid includes a Giardia-specific nuclear localization signal (NLS) to localize dCas9 to both nuclei. The schematic of the *Giardia* CRISPRi vector dCas9g1pac indicates the catalytically inactive dCas9 with a C-terminal *Giardia*-specific 2340NLS and a 3XHA epitope tag (A). dCas9 expression is driven by the *Giardia* malate dehydrogenase promoter (P_{MDH}), and a puromycin resistance marker (pac) allows positive selection in *Giardia*. The dCas9g1pac plasmid also includes inverted *BbsI* restriction sites for cloning of specific gRNA target sequences (g1 or g2) upstream of the gRNA scaffold sequence (SCF). The *Giardia* U6 spliceosomal RNA pol III promoter is used to express the gRNA cassette (g1+SCF); a non-specific gRNA is expressed unless the sequence between the BbsI sites is replaced with annealed oligomers targeting a specific genomic region. The *SacI* site can be used to add additional gRNA cassettes in tandem. Anti-Cas9 immunostaining of a *Giardia* strain carrying dCas9g1pac shows that a *Giardia*-specific native 34-amino acid C-terminal NLS from the *Giardia* protein GL50803_2340 (2340NLS) is necessary for the localization of 2340GFP to both nuclei (B). Over 50% of cells express dCas9 in both nuclei. In this strain, the microtubule cytoskeleton is visualized by expression of an integrated N-terminal mNeonGreen-tagged beta-tubulin gene selected with neomycin (mNGbtubneo). Scale bars = 5 μm.

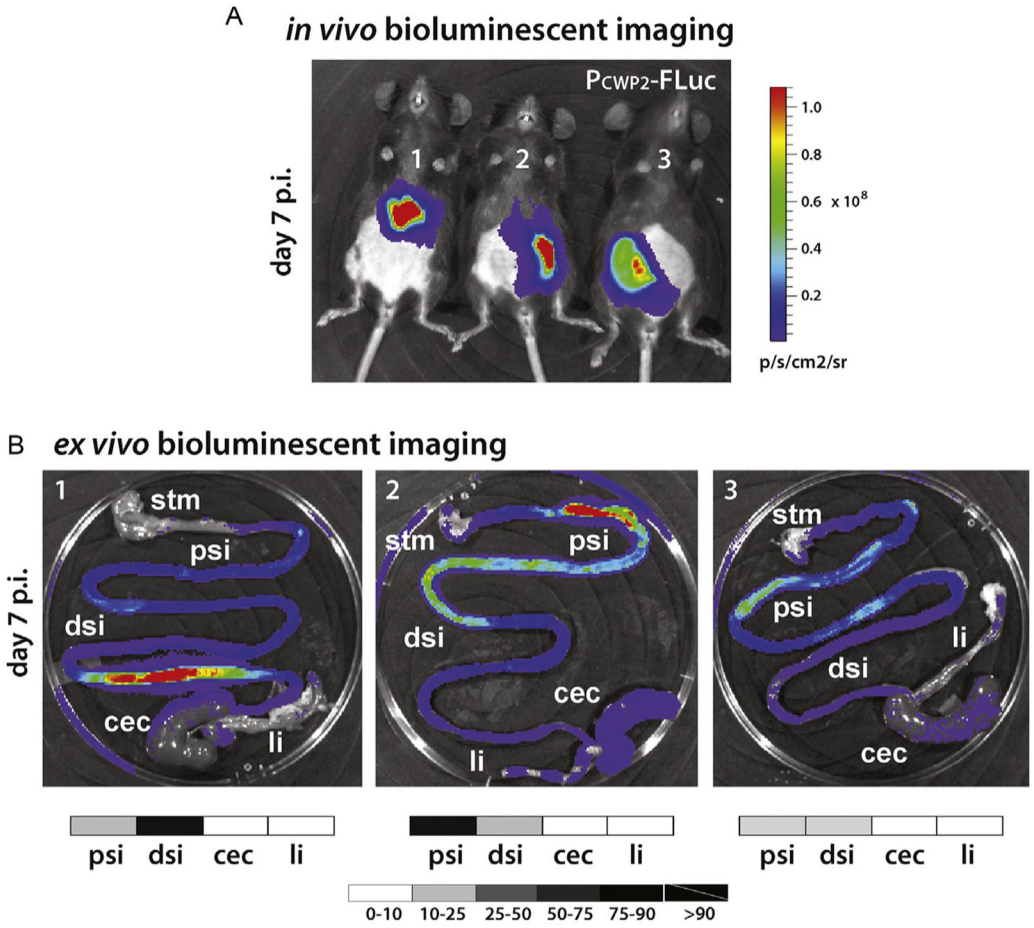

Fig. 3.

Bioluminescent imaging enables quantitative evaluation of temporal and spatial infection dynamics in the gastrointestinal tracts of small animals. Transcriptional upregulation of the *Giardia* cyst wall protein 2 (CWP2) is a genetic indicator of the initiation of encystation. Representative in vivo (A) and ex vivo (B) bioluminescent images are shown for three mice infected with the P_{CWP2}-*FLuc* strain and euthanized at 7 days post-infection (A). High bioluminescent signal is indicated for the distal small intestine (dsi) in animal 1 and for the proximal small intestine (psi) in animals 2 and 3. Photon flux or radiance (p/s/cm^2/sr) for each intestinal segment is shown and has been normalized to the maximal ex vivo bioluminescence signal on the radiance scale, yielding the percent total signal per segment. These values are represented graphically on the grey scale maps below each ex vivo image (clear = 0–10% and black = 75–100%, with values between 10% and 75% indicated as shades of grey). The regions of the gastrointestinal tract (psi = proximal small intestine, dsi = distal small intestine, cec = cecum, and li = large intestine) are noted on the ex vivo images (B). The stomach (stm = stomach) is shown for orientation but lacks bioluminescence.