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A CHEMICAL GENETICS APPROACH TO UNDERSTAND THE REGULATION OF *CRYPTOSPORIDIUM* SEXUAL DIFFERENTIATION

A Dissertation Presented

by

Md Mahmudul Hasan

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Specializing in Cellular, Molecular and Biomedical Sciences

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ABSTRACT

Cryptosporidium species are eukaryotic intracellular parasites belonging to the phylum Apicomplexa. *C. hominis* and *C. parvum* cause diarrhea in humans which is self-limiting in immunocompetent adults but can have severe consequences in immunocompromised individuals and malnourished children. In developing countries, *Cryptosporidium* is one of the leading causes of moderate to severe diarrhea in children under five years of age. There is an urgent need for novel therapeutics against this parasite as the current treatment option is inadequate to treat the most vulnerable population to Cryptosporidium infection. Better understandings of the biology of *Cryptosporidium* would greatly enhance our capability to design effective control measures.

The parasite initially multiplies through several rounds of asexual replication within the intestinal epithelial cells before differentiating into sexual forms. Here we have tested the ReFRAME library, a set of ~12,000 biologically active compounds, for activities against the asexual and sexual forms of *C. parvum* separately. We identified and validated compounds that inhibit and/or induce the growth of either or both life cycle stages. Compounds that inhibit both stages are potentially good drug candidates. Other compounds have promising tool-like properties that can be utilized to probe different aspects of *C. parvum* sexual differentiation. Several inhibitors of host cell oxidative phosphorylation and purine nucleotide biosynthesis disproportionately inhibited the sexual differentiation of *C. parvum*, highlighting the necessity of these processes in facilitating *C. parvum* sexual differentiation.

All the apicomplexan parasites differentiate into distinct forms in the course of their life cycle and each of the differentiation steps is associated with large scale changes in gene expression. To identify such stage specific genes and pathways associated with *C. parvum* sexual differentiation, we performed mRNA-seq of host cells infected with C. parvum in the presence of nine differentiation inhibitors. Ribosomal proteins were the most significantly enriched group of genes that were upregulated with multiple differentiation inhibitors, which suggested that the downregulation of these genes is associated with *C. parvum* sexual differentiation. Comparison of our results with a publicly available stage-specific mRNA-seq dataset of *C. parvum* validated this conclusion and analysis of transcriptomic datasets from other apicomplexan parasites revealed that this is a common mechanism of regulating life cycle stage differentiation in these parasites. In addition, identification of significantly enriched DNA motifs at promoters of dysregulated genes coupled with the expression pattern of several apicomplexan AP2 transcription factors strongly suggested that they play a critical role in regulating *C. parvum* sexual differentiation.

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Chris has been the most wonderful mentor that anyone can think of. Besides having all the cliched qualities of a great scientist, he has this way of becoming infectiously excited about trying out new methodologies to tackle research questions, which made my work challenging but incredibly fun at the same time. Also, I have always been amazed by his ability to quickly understand complex explanations, which I tended to do regularly, and then simplify the idea and put it into a tangible context. I am still trying to understand if it is something that can be learned or not.

I really felt blessed to have the best lab mates throughout my duration in the Huston Lab. Ze taught me most of the techniques that I used in the lab and I am yet to find a technical problem that he cannot fix. I think Rajiv's passion about our work rubbed off a little on me and made me care about my work more. Erin is the person who gets the job done, with meticulous planning and and seemingly effortless execuations. Peter

just churns out data after data from the mouse model experiments that requires working in the most uncommon hours day after day and I sometime wonder how does he get the time to do all the other things that he does in the lab. All these people were not just great lab mates, they were my mentors in many different aspects of life in USA. It was a wonderful experience to work with Ethan, Bethany and Kirtika, the latest additions to the huston lab, in my last year. All these super intelligent people brought with them much required fresh ideas and energy, and I am really excited to see where they take the lab in the next few years (and slightly sad for not to be a part of it).

I think I compiled a line up of the nicest people in UVM to be my committee members. At times, I felt like they were more sure about my ability than I ever can be and I came out from each of my committee meeting with renewed belief in myself and exciting new ideas to try. And last but not the least, I thank my wife, Tushiba, for putting up with me all this time in the midst of occasional crazy work hours and always support me when I felt down. My parents has also been a constant source of support for me and always helped me to keep my focus.

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CHAPTER 1: A REVIEW OF THE MECHANISMS THAT REGULATE STAGE DIFFERENTIATION DURING THE LIFE CYCLE PROGRESSION OF APICOMPLEXAN PARASITES

1.1: Purpose of this Review

The major goal of this thesis was to identify mechanisms underlying differentiation of the apicomplexan parasite *Cryptosporidium*. For this, we relied heavily on the main strengths of our laboratory, which are in the areas of high throughput assay development and drug screening. We describe development of a microscopy-based screening assay for asexual to sexual stage development of *Cryptosporidium*, its use to screen a library of biologically active small molecules, and then follow up studies to characterize the effects of identified inhibitors using RNA-seq. This literature review chapter provides background on parasites related to *Cryptosporidium* within the phylum Apicomplexa, what is known about differentiation of major apicomplexan parasites, and general themes that have emerged from prior research on apicomplexan differentiation.

1.2: Origin and Classification of Apicomplexan Parasites

Apicomplexa is a phylum of unicellular eukaryotes that are obligate intracellular parasites. The defining characteristic of this phylum is that they all harbor an apical complex, a structure composed of specialized organelles and cytoskeletal

structures, during at least one stage of their life cycle. It is present at the anterior end of the parasite and is required to invade the host cell. Apicomplexans infect almost all vertebrates and invertebrates and the host specificity of each species is relatively restricted, with some notable exceptions (1-3).

Another feature of apicomplexans beside the apical complex is the apicoplast, a non-photosynthetic plastid, that is present in most of the apicomplexan parasites. The apicoplast genome has given us major clues about the origin and evolution of apicomplexans. The four membrane enclosure of the apicoplast suggests a secondary endosymbiotic origin of the organelle (4). Such an organelle is, however, not completely unique in apicomplexans. Around half of the members of Dinoflagellata, a sister phylum of Apicomplexa consisting of unicellular planktons, are among the other groups of organisms that contain a secondary endosymbiotic plastid (4). Interestingly, even though phylogenetic analysis of plastid rRNAs shows that apicomplexan and dinoflagellate plastids came from the same origin, their gene sets do not overlap a lot (5). Discovery of the unicellular photosynthetic organisms *Chromera velia* and *Vitrella brassicaformis* provided the missing link between apicomplexans and dinoflagellates (6, 7). Both of them have a secondary endosymbiotic plastid that encode a superset of genes present in the plastids of apicomplexans and dinoflagellates. Phylogenetic analysis of their plastid genome reinforced the already widely supported notion that their plastid, just like the plastids of apicomplexans and dinoflagellates, originated from red algal plastids (6). Phylogenetic trees constructed using the 18S rRNA and nuclear

genes also point towards a common ancestor of apicomplexans, dinoflagellates and chrompodellids (*Chromera velia*, *Vitrella brassicaformis,* and other related genera) (8, 9). Therefore, the currently held view is that the common ancestor of all three above mentioned groups was a free-living photosynthetic unicellular eukaryote with a secondary endosymbiotic plastid of red algal origin. Subsequent evolution of this ancestor gave rise to several lines, some keeping the photosynthetic capability while apicomplexans lost it (10, 11). Importantly, there are groups of organisms in different lineages originating from this ancestor that have also completely lost the plastid (9).

Transfer of genes between organelle genome and nuclear genome, termed endosymbiotic gene transfer, is very common in eukaryotes, including apicomplexans (12, 13). A class of transcription factors (TFs), the Apetala 2 (AP2) domain-containing TFs, exemplify such a mode of gene transfer between apicoplast and nuclear genome. Several apicomplexans severely lack common TF families normally present in animal cells (14). In silico identification of DNA binding proteins followed by searching their homologs in all life forms identified that several apicomplexan DNA binding proteins share homology to plant Apetala 2 domain-containing transcription factors (14). The apicomplexan orthologues are named Apicomplexan AP2 (ApiAP2) TFs. Genomes of all apicomplexans, as well as their apicoplast bearing cousins sequenced so far, have this family of TFs in their genome, some more than the others (14, 15). This suggests that after an initial endosymbiotic transfer, this family of TFs later expanded in a lineage-specific manner (14). The role of ApiAP2 TFs in apicomplexan gene

regulation has been studied extensively and several of them play key regulatory roles in the progression of the apicomplexan life cycle.

Apicomplexans can be broadly divided into four different groups based on phylogenetic analyses, structural features and host range (1, 2, 16, 17). The most understudied among them are the gregarines, which are parasites of invertebrates. Advances in environmental DNA sequencing are slowly unearthing an abundance of gregarine parasites in diverse environments; however, molecular characterization of the members of this group is scarce (8, 18, 19). Phylogenetically, gregarines are difficult to group together among themselves, leading to them being called deep-branching apicomplexans (8, 9). The apicoplast is presumed to be lost in many gregarines (9). Members of the sole genus *Cryptosporidium* constitutes the second major group of apicomplexans. These are parasites of the vertebrate gastrointestinal tract. None of the *Cryptosporidium* species harbors an apicoplast and they have also lost a fully functional mitochondrion. Instead, they have a mitochondrion-like organelle called a mitosome that lacks its own genome. Several nuclear-encoded genes are nonetheless targeted to this relict mitochondrion and its primary function seems to be Fe-S cluster generation (20-22). Until recently, *Cryptosporidium* species were considered to be members of another major apicomplexan group called the coccidians (17). Coccidians are parasites of both vertebrates and invertebrates. Their similarity to *Cryptosporidium* is that they produce cysts that are the infective form of the parasite. However, unlike *Cryptosporidium*, they have functional apicoplasts and mitochondria. Coccidians are

very diverse in their host range; some of them are specific for certain host species, while others have a broader host range (23, 24). The most well-studied coccidian parasite is *Toxoplasma gondii*, which is often used as a model apicomplexan due to the availability of well-established genetic manipulation systems for this parasite. Finally, the other group of apicomplexans, haematozoa, are parasites of the vertebrate circulatory system that are transmitted by blood-sucking insect vectors. The insect vectors are the definitive hosts of this group of parasites, as sexual replication occurs within them, while vertebrates are intermediate hosts where they replicate asexually. The apical complex of haematozoans lacks a conoid, a special arrangement of microtubules, that is present in the apical complex of all the other groups of apicomplexan parasites (25, 26). *Plasmodium* parasites, the causative agents of malaria, belong to this group.

1.3: Significance of the Research on Apicomplexan Stage Differentiation and Scope of this Review

One of the common aspects of the life cycle of all the apicomplexan parasites is that they differentiate into distinct forms at various stages of their life cycle. All of them have at least three different forms, namely, sporozoites, asexual replicative forms, and sexual replicative forms. The term for asexual replicative forms varies by species. There is no sex chromosome in apicomplexans, but mature gametes are dimorphic. Most commonly, one gamete is comparatively larger than the other gamete, which is

called the macrogamete or the female gamete. The comparatively smaller gamete, which is usually formed from a gametocyte containing multiples of them, is called the microgamete or male gamete. A gamete within a host cell is called a gametocyte. Additionally, based on the species, they take other special forms through the progression of their life cycle.

Apicomplexan differentiation has been an active area of research. The fact that each of these unicellular intracellular parasites can transform into very different forms characterized by altered structures and phenotypic characteristics, as well as different modes of replication and metabolism, while maintaining essentially the same genetic makeup makes apicomplexan parasites ideal candidates to study the regulation of gene expression and basic cell biology. From an evolutionary and ecological perspective, how apicomplexans survive in diverse conditions and each establishes its own ecological niche are of huge interest.

Several apicomplexans cause significant diseases in humans that are of major public health and economic concern. *Plasmodium* parasites cause malaria, which still kills thousands of people every year. Blocking *Plasmodium* differentiation in people is central to transmission-blocking strategies (27). Around one third of the population in the world are thought to be chronically infected with *T. gondii gondii*, an opportunistic pathogen of humans. The secret behind this astonishingly high prevalence is its ability to differentiate into a cyst form, which is untreatable with the current therapeutics (28). *Cryptosporidium* has recently been identified as one of the major causes of childhood

diarrhea in the developing world (29). There is no efficacious therapeutic available for *Cryptosporidium* infection of immunocompromised patients and malnourished children, the two most vulnerable populations to *Cryptosporidium* infection (30). Our current understanding of *Cryptosporidium* biology suggests that blocking differentiation would be an effective therapeutic strategy against this parasite.

Early development of apicomplexan parasites within the human body involves differentiation from the environmentally resistant form or vector transmissive form to a more human adopted form capable of establishing an infection. Each of the pathogenic Apicomplexa subsequently differentiate further to rapidly replicating forms that are more associated with the pathologies of the diseases they cause. While inhibiting the later stages are straightforward strategies in drug development, understanding the early stages are more important towards vaccine development. Development of attenuated strains incapable of early differentiation as well as using early stage-specific cell surface markers as subunit vaccines are two of the most common strategies employed in anti-apicomplexan vaccine development (31, 32).

Unsurprisingly, detailed studies on the molecular mechanism of differentiation have been mainly performed on apicomplexan genera that are pathogens of human or economically important pathogens of livestock and poultry. In this review, we will discuss the differentiation of three haematozoans (*Plasmodium, Babesia, Theileria*), two coccidians (*T. gondii* and *Eimeria*) and the sole genus of the group *Cryptosporidium*. Notable pathogens that are not discussed due to relative scarcity of

differentiation-related literature include two coccidian parasites, the human pathogen *Cyclospora* and the livestock pathogen *Neospora*. No gregarine parasite has been discussed, as their molecular mechanism of differentiation has not been studied yet (33).

A key aspect of the apicomplexan differentiation processes is the extensive change in the global gene expression pattern associated with differentiation. Emphasis has been given in this review about characterizing these global changes, as well as key components that play master regulatory roles in bringing about these changes. However, numerous studies have also been performed to understand the structures and functions of components that have roles in the developmental progression of each of the distinct differentiated stages. Several comprehensive reviews have been published discussing such components for a particular stage of each of the parasites' life cycles. Instead of repeating the same information in this review, we have referred to those articles wherever appropriate.

1.4: *Plasmodium* Stage Differentiation

Plasmodium parasites are the causative agents of malaria, which killed more than 400 thousand people in 2018. Vertebrates are the intermediate hosts and mosquitos are the definitive hosts of *Plasmodium* species. Six *Plasmodium* species infect humans, among them *P. falciparum* is the most well-studied species. Several simian, avian and rodent *Plasmodium* species are used as model organisms in

laboratory settings to understand its biology (34). Notable among them are the rodent parasites *P. berghei* and *P. yoelii*. Continuous in vitro cultivation of several species of *Plasmodium* is performed within red blood cells (RBCs) (35).

1.4.1: Sporozoite to Liver Stage Differentiation

Sporozoites, the form of *Plasmodium* parasites that initiates infection of the vertebrate host, encounter the most diverse conditions among all the life cycle stages of *Plasmodium* (Figure 1). They are developed inside oocysts residing at basal lamina of the mosquito midgut, and after egressing from the oocyst, they travel via hemolymph to the mosquito salivary gland and invade it. Salivary gland sporozoites are inoculated into vertebrate skin during a mosquito bite, traverse the dermis to gain entry into the blood circulation, and then, in mammalian hosts, sporozoites again traverse the endothelial cells in liver sinusoids to access hepatocytes where they can start the exoerythrocytic phase of the infection (36, 37). It is not surprising that sporozoites at different stages of this process differ from each other. Many *Plasmodium* genes have been identified that are essential for a specific part of the sporozoite's life while dispensable for the others. It suggests a model of stepwise maturation of sporozoites capable of establishing infection in the liver (36, 37).

Interestingly, commitment to the exoerythrocytic phase seems to be attained even before the sporozoites contact the vertebrate host. First shown in the rodent parasite *Plasmodium berghei*, sporozoites from the salivary gland are 10,000 times more infectious to the mammalian host than sporozoites from oocysts (38). The same

phenomenon was subsequently shown in several other *Plasmodium* species, and, with the avian parasite *P. gallenaceum*, it was shown that salivary gland sporozoites lose infectivity for the mosquito salivary gland once they were reintroduced into the mosquito hemocoel (39). Sporozoites in the hemolymph show intermediate infectivity and other intermediate phenotypic characteristics that fall between the characteristics of salivary gland sporozoites and oocyst sporozoites (40).

Comparative transcriptomics and proteomics analyses between oocyst sporozoites and salivary gland sporozoites to determine the regulation of this commitment have identified multiple genes upregulated in one population compared to others (41-44). Knockout of several of the genes upregulated in infectious sporozoites (UIS) formed parasites that are capable of infecting hepatocytes, but then fail to successfully replicate inside them (45-49). The most studied among them, which has often been used as a marker for salivary gland oocyst and liver stage *Plasmodium*, is UIS4 (45). UIS4 regulation in sporozoites and liver stage *Plasmodium* provides important mechanistic details about *Plasmodium* sporozoite to liver stage differentiation (45). Even though UIS4 mRNA is highly expressed in salivary gland sporozoites, it is post-transcriptionally repressed in sporozoites and the protein level eventually goes up in the liver stage (50). The RNA binding protein Puf2 binds to the UIS4 transcript and keeps it in a repressed state (51). Puf2 knockout sporozoites reach the salivary gland, but cannot infect the liver (47, 48, 52). This mutant also differentiates into a form that resembles early liver stage *Plasmodium* while still

residing in the mosquito salivary gland (47, 48, 52).

Several studies suggest that this transcriptionally active but translationally repressed state is not a unique feature of UIS4, and that this is a general strategy of *Plasmodium* to be prepared for infection upon switching hosts (44, 48, 53, 54). Indeed, the kinase IK2 that phosphorylates and inactivates translation initiation factor eIF2α has been shown to be an important regulator of this process in *Plasmodium (54)*. IK2 knockout parasites showed reduced phosphorylation of eIF2α and an overall upregulated protein translation, including translation of UIS4, in sporozoites (54). Phenotypically, the IK2 knockout mutant also prematurely develops into a liver stage like form (54). Translationally repressed UIS mRNAs, including UIS4, are actively protected from degradation in salivary gland sporozoites, and a *Plasmodium* protein called SAP is involved in that process (49). Like the other mutants mentioned before, SAP knockout also inhibits liver stage development (49).

Recently, RNA-seq based transcriptomics, quantitative proteomics, and high throughput screening of knockout mutant libraries have verified some of those earlier findings, while also generating a clearer landscape of metabolic changes during differentiation (55-58). For example, a mutant library screening demonstrated essentiality of the type II fatty acid biosynthetic pathway in liver stage development (58). Some of the members in this pathway are upregulated in salivary gland sporozoites, and were previously shown to be essential in parasite development into the liver stage (59, 60). Another study performed comparative RNA-seq and proteomics

analyses of oocyst sporozoites and salivary gland sporozoites of both *P. yoelii* and *P. falciparum*, and validated the translational repression program in salivary gland sporozoites (57). Interestingly, this study also suggests an independent translational repression program in oocyst sporozoites that is released after sporozoite invasion of the salivary gland (57). This might be the explanation for the inability of salivary gland sporozoites to reinfect the salivary gland as mentioned before (39). Follow up genetic experiments based on these high throughput sequencing analyses (57) would likely help in identifying genes and pathways critical for differentiation.

The stimulus for sporozoite to liver stage differentiation has been suggested to be dependent on time (i.e. after a certain time, a sporozoite is committed to differentiation) and space (i.e. the environment around a sporozoite determines its stage of commitment); however, no definite molecular signal has been established yet. In terms of a transcriptional regulator, AP2-L is an AP2 transcription factor that is necessary for liver-stage development (61-63). AP2-L protein is expressed in blood stage, sporozoite and liver-stage *P. berghei*, but the AP2-L knockout mutant showed complete developmental arrest only in Liver stage (61). Knockout mutants of three other AP2 TFs in *P. berghei* failed to form sporozoites from oocysts, or the sporozoites failed to reach the salivary gland (62-64). Detailed identification and characterization of the targets of these transcription factors would likely provide more information about the differentiation of sporozoites.

1.4.2: Liver Stage to Erythrocytic Stage Differentiation

The exoerythrocytic cycle for mammalian *Plasmodium* species takes place within hepatic cells (Figure 1). A sporozoite first transforms into a trophozoite, which then replicates into thousands of merozoites within a single hepatic cell. Packets of merozoites called merosomes bud out from the hepatic cells into the blood, and are ultimately released to start the erythrocytic cycle of *Plasmodium* infection (65, 66). Numerous individual proteins are implicated at different stages of this development process through single-gene knockout studies (nicely summarized in Table 1 of (67)). However, identification of master regulatory elements involved in exoerythrocytic to erythrocytic stage differentiation is still absent. One potential reason for this is the scarcity of transcriptomic and proteomics data in high enough temporal or phenotypic resolution as to differentiate different stages of the exoerythrocytic phase. Fortunately, such experiments are starting to come out (55, 56). Another reason that is likely hampering the characterization of master regulatory elements is the standard genetic manipulation system of *Plasmodium*. The erythrocytic phase parasite has largely been the starting point of genetic modification and clonal selection in *Plasmodium* (68). As such, essential genes for erythrocytic growth are not suitable for straight knockout. The liver stage directly precedes the erythrocytic phase, and it is highly likely that factors involved in differentiation from liver stage to the erythrocytic stage are also essential during erythrocytic schizogony. In addition, knockout of any gene that is also essential in the sexual and/or sporozoite stage would never produce viable liver stage

Plasmodium. Therefore, targeted analysis of any gene function in liver stage *Plasmodium* with the aid of a knockout mutant of that gene would only be possible if the gene is not essential in any other stages of the *Plasmodium* life cycle. The development of several conditional knockout systems in *Plasmodium* has huge potential to overcome this limitation (69, 70).

Several *Plasmodium* species, including the human pathogen *P. vivax,* differentiate into a latent form termed a hypnozoite after infecting liver cells (71). A hypnozoite culture system and omics data have just started to emerge in the past few years (72-74), but the induction and relapse mechanism is still largely unknown.

1.4.3: Sexual Differentiation of *Plasmodium*

Plasmodium sexual differentiation is one of the most heavily studied differentiation processes among apicomplexans. The merozoites from the liver infect RBCs to start the erythrocytic cycle of *Plasmodium* infection. Upon infection, the parasite takes on a flat disk-like morphology called a ring phase, then it rounds up to form a trophozoite, which then replicates multiple times through erythrocytic schizogony to produce up to 32 merozoites that are released into the blood after RBC rupture. The merozoites start another cycle of RBC infection, and either repeat the asexual cycle again or develop into gametocytes. RBCs enclosing mature gametocytes circulate in the bloodstream until taken up by a mosquito (Figure 1). Gametocytogenesis, the process of developing mature gametocytes, takes 24 to 60 hours in most *Plasmodium* species and 9 to 12 days for *P. falciparum* (34, 75).

Interestingly, the commitment to gametocytogenesis occurs one schizogony cycle before the ultimate gametocytogenesis. Seminal experiments, first with Giemsa staining and then with stage-specific antibody staining of *Plasmodium* on RBC monolayers demonstrated that all the progeny merozoites from a single schizont either develop into gametocytes or asexual merozoites (76, 77). Therefore, even though the schizonts are morphologically indistinguishable, they are already committed to form either sexual or asexual forms. Subsequently, using gender-specific antibody staining, it was shown that a sexually committed schizont gives rise to either male or female gametocytes exclusively (78, 79).

Several laboratory clones of *Plasmodium* do not form gametocytes. Comparative genomic analysis of these clones followed by targeted gene knockouts identified two master regulators of this commitment, AP2-G and GDV-1 (80-82). AP2-G, a member of the AP2 transcription factor family, is expressed in the sexually committed schizonts and acts as a transcriptional activator for several early gametocyte genes (81, 82). In *P. falciparum*, the expression of AP2-G itself is under the control of heterochromatin protein 1 (HP-1), which epigenetically represses the expression of AP2-G by binding at the ap2g locus (83). GDV-1 has been shown to interact and evict HP-1, and thus release the transcriptional repression of AP2-G (84). This puts GDV-1 upstream to AP2-G in terms of gametocytogenesis regulation. This epigenetic mode of gametocytogenesis control is well supported by other studies. For example, several histone post-transcriptional modifications are exclusively enriched during

gametocytogenesis (85), and several epigenetic modifiers selectively inhibit the sexual and asexual stages of *Plasmodium* (86).

A forward genetic approach using *piggyBac* transposon mutagenesis identified 16 genes essential for gametocytogenesis (87). Targeted analysis of AP2 gene family members in *P. berghei* showed that knockout of AP2-G2, a transcriptional repressor, results in a developmental arrest of gametocytes following commitment (88), and knockout of AP2-FM, potentially a transcriptional activator, results solely in a failure of female gametocyte development (89).

Comparative genomics, mutant library screening and targeted mutagenesis approaches led to the identification of several other *Plasmodium* genes that have roles in general gametocytogenesis, as well as male and female gametocyte production (Table 1 in (90), (34)). However, comparative transcriptomics is arguably a more powerful tool to understand the dynamic genetic regulation of the differentiation process. Microarray analysis of tightly controlled timepoints has revealed a picture of cyclic changes in the gene expression pattern in *Plasmodium* during erythrocytic schizogony (91). However, similar transcriptomic or proteomic comparison between sexual and asexual stages of *Plasmodium* is challenging, because gametocytogenesis is a rare event during the erythrocytic replication stage of *Plasmodium*, which makes it difficult to capture clean '-omics' data from gametocytes using simple time point resolutions (92, 93). This challenge has been partially met by FAC (Fluorescent activated cell) sorting transgenic parasites expressing fluorescent proteins under male

or female specific promoters (94-96). It allowed the identification of male and female specific proteins and pathways in *Plasmodium* (94-96). However, male and female gametocytes are comparatively late stages of the gametocytogenesis process in which the sexual conversion has already taken place. But as mentioned before, the commitment to those stages occurs earlier in a sexual/asexual indistinguishable schizont stage.

There is still no clean early sexually committed schizont marker. Single cell transcriptomics has proven to be a particularly well suited technique to address this challenge, as it allows the analysis of transcriptome from rare cell populations. The first single cell transcriptomic experiment focusing on sexually committed schizonts identified, among other things, two additional AP2 transcription factors and four chromatin modifiers differentially upregulated in sexually committed schizonts, reaffirming the role of AP2 TFs and epigenetics in *Plasmodium* sexual commitment (97). It also described a more detailed expression pattern of AP2-G. AP2-G is expressed in a low level in both early sexually and asexually committed schizonts. Following a trough in expression in both populations, it is expressed highly only in mature sexually committed schizonts (97). Since then, other studies have used this technique to characterize the early stages of gametogenesis, and implications and validation of their findings would surely paint a clearer picture of sexual commitment in *Plasmodium* (84, 98). Importantly, one of them reported that gametocytogenesis can happen without a committed schizont stage, which results in the formation of mixed

plaques containing both sexual and asexual merozoites, questioning the decade-old standard theory of gametocytogenesis commitment (98). The proposed theory behind this mechanism is in line with the detailed temporal AP2-G expression pattern mentioned before. The study proposes that in early schizonts, if AP2-G expression reaches a threshold, it allows AP2-G to express again in mature schizonts, committing them to gametogenesis. In support of this theory, they showed that artificial AP2-G stabilization during schizogony can induce gametogenesis without going through a committed schizogony cycle (98).

Stress on both host and parasite has long been considered to be a major signal for gametocytogenesis (99). This belief radiates from the observation that high doses of anti *Plasmodium* drugs increase the rate of sexual conversion (100) and that *P. falciparum* gametocytes are preferentially developed in reticulocytes, which are immature erythrocytes that are more readily available in anemic blood (101, 102). Based on several empirical datasets that analysed the relationship between stress and *Plasmodium* sexual conversion, a model proposed that reducing gametocyte conversion during low levels of stress actually increase the chance of future successful transmission whereas under highly stressed condition, the parasite allot more resources to gametocytogenesis as a way of terminal investment for successful transmission. High drug pressure is such a stress condition for the parasite when mortlity rate exceeds the parasite proliferation rate or severe anaemia is such a stress condition of the host when the mortality is imminent (99). Recently, depletion of a serum factor called

lysoPC has been shown to be a signal for gametocytogenesis in *P. falciparum* but not in *P. berghei* (103). The relationship of this molecule to stress is unclear. In any case, this aspect of gametocytogenesis requires further focus.

1.4.4: Sex-specific Maturation for Differentiation into Zygote

The gametocytes are taken up by the mosquito during a blood meal. In the mosquito midgut, gametocytes are activated to form gametes, actively come out of the RBC, and mate to form the zygote (Figure 1). Gamete activation or gametogenesis involves rounding up of the gametocyte, disrupting the RBC membrane, and the egress of mature gametes in the mosquito midgut. Change of temperature and pH from mammalian blood to mosquito midgut, as well as the mosquito derived factor xanthurenic acid, act as signals for gametogenesis (104, 105). A meiotic nuclear division without diakinesis occurs within three hours of zygote formation, and the diploid zygote transforms into a motile tetraploid ookinete (106). The ookinete traverses the mosquito midgut epithelium to reach the basal lamina, transforms into an immotile oocyst, and multiple haploid sporozoites are formed. The initial stages in the mosquito vector are quite rapid; within 15 minutes the gametes come out, and the zygotes are formed within two hours in the mosquito midgut. Ookinete development takes 20 h to 35 h, and the oocyst development is a long process, taking around 10 to 12 days (107).

Two distinct maturation programs run in parallel in male and female gametocytes, culminating in male and female gamete formation at the same time and

space. Multiple proteins have been characterized that are expressed differentially in male and female gametocytes and are essential at different stages of development of *Plasmodium* gametocytes in a sex-specific manner. Knockout of these genes usually results in a deficiency in gametocyte maturation/ gamete activation/ zygote formation/ ookinete conversion or dysfunctional ookinete formation (Table 1 in (107), (108-110)). Indeed, global transcriptomic and proteomic comparisons of male and female gametocytes uncover distinct expression profiles for them that are shaped by their distinct needs (94-96). For example, upon activation, a male gametocyte divides mitotically three times to produce eight microgametes. The motile microgametes lyse and egress from the RBC in a distinct fashion called exflagellation. The formation and motility of microgametes require assembly of an axoneme, a microtubule-based structure. The transcriptome and proteome of male gametocytes are overwhelmingly enriched for DNA replication and axoneme formation machinery compared to the female gametocyte (94, 96). On the other hand, the cytoplasm of zygote and early ookinete is derived from female gamete, as the smaller male gametes mostly only supply a copy of the genome to the female gamete during fertilization. The zygote and early ookinete express a lot of new proteins, which is a hallmark of the stage conversion (111, 112). To support this rapid transformation, a lot of cellular energy is also required. In line with this point of view, the female gametocyte transcriptome and proteome are enriched for pathways of protein synthesis and degradation, as well as energy metabolism (94-96).

The phenomenon of "just in time" expression observed for the mosquito to mammalian transition in *Plasmodium*, where mRNAs overexpressed in salivary gland sporozoites are kept in a translationally repressed form that are to be expressed upon host switch, is also observed in female gametocytes during host switch. An RNA helicase, DOZI (development of zygote inhibited) was found to be important for this process in *P. berghei* (113, 114). DOZI knockout female gametocytes produced normal female gametes and zygotes; however, zygote to ookinete differentiation was halted before meiosis (114). DOZI is localized with CITH and 14 other core components to a cytoplasmic assemblies called P granules (113). These granules protect overexpressed but untranslated mRNAs in female gametocytes. In DOZI knockout female mutants, those mRNAs are degraded (113, 114). DOZI knockout male mutants produce ookinetes normally, suggesting this translational repression is a female-specific program (114). A wide variety of mRNAs including known transcription factors required for maturation of ookinetes as well was ookinete cell surface markers required for diverse ookinete functions are protected by these P granules (113). A matched transcriptomics and proteomics analysis of female gametocytes corroborates the existence of this phenomenon, where a large number of protein products from overexpressed mRNA are undetectable in female gametocytes (94). Gene ontology enrichment analysis on putatively transcriptionally repressed genes in female gametocytes identifies meiosis as the highest enriched biological process, which is consistent with ookinete developmental arrest before meiosis in

female DOZI knockout mutants (94, 114).

The energy metabolism of male and female gametocytes is also divergent. Chemical inhibition or genetic disruption of the TCA cycle and oxidative phosphorylation is very deleterious for *Plasmodium* in mosquito stages but well-tolerated in the asexual blood stage, as glycolysis is the overwhelmingly major source of energy in RBC stage *Plasmodium* (115, 116). The gametocytes, however, show an intermediate phenotype. Gender independent analysis of the proteome of gametocytes suggests the presence of the TCA cycle and ETS components in them (117). Male gametocyte activation, usually measured through the exflagellation rate of male gametocytes, is affected by chemical inhibition of glycolysis while female gamete activation remains unaffected (116, 118). If components of oxidative phosphorylation are knocked out, the female gametocyte development and zygote to ookinete transformation are disrupted, but male exflagellation is unhampered (119, 120). Taken together, it suggests that male gametocytes depend on glycolysis-based energy production, just like asexual *Plasmodium*, but the female gametocytes are not completely dependent on glycolysis and start to shift towards mitochondrial energy production as used by the ookinete stage.

Importantly, male and female gametocyte maturation and release are synchronized in space and time, so that both male and female gametes can come out together in the mosquito midgut and find each other. Therefore, even though the gametes develop differently, they most likely respond to the same or similar

extracellular stimuli, using similar intracellular components. For example, calcium and cGMP signaling plays critical roles in the egress of both gametes (107, 121). Chemical inhibition, as well as genetic disruption of cGMP-dependent protein kinase PKG and cGMP phosphodiesterase, causes inhibition of both male and female gamete activation (121-123). Conditional disruption of calcium-dependent protein kinase CDPK1 leads to delayed microgamete emergence from male gametocytes, as well as a severe arrest in ookinete development (124, 125). Molecular characterization of the later phenotype showed that CDPK1 activity acts as a switch to turn off the transcriptional silencing program in female gametocytes (125).

1.4.5: Zygote to Ookinete, Ookinete to Oocyst, and Oocyst to Sporozoite differentiation

Global transcriptomic and proteomic comparison between zygote and ookinete is inherently difficult, as gametocyte to ookinete transformation occurs very rapidly. A few studies where this aspect of differentiation was analyzed reported the presence of several enriched mRNAs and proteins in ookinetes stages with potential function in invasion and metabolism (111, 112). Several gene knockouts in *Plasmodium* resulted in ookinete maturation defects, but a substantive number of them are also transcribed specifically in female gametocytes, suggesting they are likely to be regulated via the P body mediated translational repression program (107, 126, 127). For example, the most

commonly used ookinete markers, P25 and P28, are important for ookinete maturation and function (128). Their transcription occurs in female gametocytes, but they are translated only after the transcriptional repression program is turned off (114).

The differentiation of ookinete to oocyst is scarcely studied in *Plasmodium*, and recent data suggest that large scale transcriptional differences exist between these two stages (129). Targeted disruption of several AP2 transcription factors resulted in dysfunctional ookinete or oocyst formation (62, 63). However, temporal regulation of these mutants along the ookinete development trajectory has not been studied in fine detail.

Targetted analyses of AP2 TFs in P. berghei and P. yoelii suggest that a continuous spectrum of progression from female to ookinete to oocyst is controlled by different AP2 TFs (62-64, 89). For example, in Plasmodium berghei, AP2-FG is required for female gametocyte maturation, AP2-O1,2,3,4 are required for ookinete maturation and oocyst establishment, and AP2 SP1,2,3 are required for oocyst maturation to form sporozoites (62). In P. yoelii, another putative TF, HMGB2, was found that regulates ookinete maturation (130). An AP2 TF specifically required for male gametogenesis has not been described yet.

1.5: *Toxoplasma gondii* Stage Differentiation

T. gondii gondii has extensively been used as a model apicomplexan parasite due to relative ease of in vitro culture, earlier development of genetic tools, and

availability of well-established animal models compared to other apicomplexans (131). It is reported to be capable of infecting any nucleated animal cell, and is routinely cultured in human foreskin fibroblast (HFF) cells in laboratory settings (132). Warm-blooded animals are intermediate hosts of the parasites, which become infected by ingestion of oocysts (found in the feces of cats, the definitive host) or tissue cysts residing in the tissues of an animal (Figure 2). In the gut, tissue cysts release merozoites that infect intestinal epithelial cells where they asexually replicate by endodyogeny, a special form of replication characterized by forming two daughter cells within a mother cell and ultimately consuming it. Asexual replication usually gives rise to eight tachyzoites that lyse the host cell and infect nearby cells. If tachyzoites gain access to leukocytes, they migrate to different tissues. In immunocompetent hosts, tachyzoites ultimately differentiate into bradyzoites, a slowly replicating quiescent form that is subsequently enclosed by a cyst wall within the host cell. The number of bradyzoites in the tissue cysts range from two to hundreds. This encystation process establishes a lifelong asymptomatic chronic infection of *T. gondii*. In the case of immune suppression of the host, e.g. by infection or chemotherapy, tissue cysts can be reactivated and the bradyzoites differentiate back into tachyzoites. Eating raw meat containing tissue cyst disseminates the infection to a new host (28).

1.5.1: Tachyzoite-Bradyzoite differentiation

1.5.1.1.: Lessons from the Induction Signals

Stress has been established as the key environmental stimulus for tachyzoite to bradyzoite differentiation. It can be applied in various forms like temperature or P^H change, drug administration, activation of the host immune system, and metabolic inhibition (133). Interrogation of how such diverse stresses induce the same phenotype of tachyzoite to bradyzoite differentiation led to the characterization of eIF2α (eukaryotic Initiation Factor 2 alpha) phosphorylation, a known marker of the eukaryotic stress response, in *T. gondii* (134). eIF2α is a highly conserved transcription initiation factor in eukaryotes that is repressed by phosphorylation (135). Several above-mentioned stressor classes induce phosphorylation of eIF2α in *T. gondii*, and this phosphorylation mark is kept in bradyzoites, suggesting a link to this phosphorylation to bradyzoite differentiation (136). Chemical inhibition of phosphorylation led to reduced bradyzoite conversion, and also reduced tachyzoite lytic proliferation both in vivo and in vitro (137). On the other hand, inhibition of eIF2α dephosphorylation increases the bradyzoite conversion rate and inhibits the reactivation of bradyzoites (138). These data indicate that eIF2α phosphorylation is necessary for bradyzoite differentiation. Four classes of eIF2α kinases have been identified in *T. gondii* that phosphorylate eIF2α in under distinct stress conditions (134, 136, 139-141). Gene knockout of two of these kinases inhibits eIF2α phosphorylation and the mutant tachyzoites showed reduce infectivity in in-vitro cell culture after specific stress

inductions (139, 140). Replacing the wild type eIF2α with a phosphorylation deficient eIF2α phenocopies both of the kinase knockouts (139, 140, 142). Additionally, death is delayed in mice infected with these mutants (139, 140, 142). Therefore, eIF2α phosphorylation is also important for the lytic tachyzoite. In several other systems, phosphomimetic versions of eIF2α have been described (143-146). In *T. gondii*, such mutant eIF2α expression has not been described yet. It would be interesting to observe if phosphomimetic eIF2α expression is possible in *T. gondii* and if yes, would it be sufficient to drive tachyzoite to bradyzoite differentiation.

Several stressors that induce bradyzoite differentiation hamper mitochondrial function. Atovaquone, a drug that targets apicomplexan mitochondrial activity, was found to be ineffective against a clone of *T. gondii* that spontaneously differentiates into bradyzoites (147). It led to the testing and validation of atovaquone and some other drugs that supposedly act on mitochondria to be inducers of bradyzoite differentiation (147). IFNγ, another inducer of bradyzoite differentiation, induces macrophages to produce nitric oxide, which in turn reacts with Fe-S clusters of the mitochondrial respiratory chain, rendering the organelle dysfunctional (148). When bradyzoite and tachyzoite extracts were tested for the enzymatic activity of several glycolytic and TCA cycle enzymes, bradyzoite extracts showed higher activity for glycolytic enzymes (149). Recently, it has been shown that knocking out hexokinase, the first enzyme in the glycolytic breakdown of glucose, severely blocks bradyzoite formation in mouse brain, but only modestly affects lytic replication of tachyzoites

(150). Isotope-labeled metabolite tracking showed that tachyzoites can use glutamine in the TCA cycle to generate energy in the absence of glucose utilization, and also synthesize glycolytic intermediates from gluconeogenic pathways (150). Taken together, all these observations suggest that mitochondrial respiration is important for tachyzoite energy production, but not so critical for bradyzoite energy production.

One bradyzoite inducing compound, called compound 1, greatly exemplifies the role of the host cell on bradyzoite differentiation (151). This compound was initially identified as an inhibitor of cGMP-dependent PKG (152, 153). Interestingly, pretreatment of HEF cells with compound 1 resulted in the induction of bradyzoite differentiation (151). Microarray analysis of host cells revealed that the expression of cell division autoantigen-1 (CDA1) is 47-fold upregulated in response to compound 1 treatment. RNAi-mediated silencing of CDA1 rendered parasites insensitive to compound 1, whereas overexpression of CDA1 induced differentiation similar to the level of compound treatment (151). CDA1 can inhibit cellular growth, and also is implicated in tumorigenic and DNA damage response pathways in mammalian cells (154). Indeed, cell types that do not grow rapidly, like terminally differentiated neurons or muscle cells, are more permissive to bradyzoite cyst formation (155, 156). The molecular mechanism of how the host cell's replicative potential is transmitted to the parasite is still unknown. Different cell types respond differently in terms of their global gene expression pattern and common pathways have not been identified that can robustly explain the host cell-type tropism of bradyzoite differentiation (155, 157).

1.5.1.2: Lessons from the -Omics Studies

Tachyzoites and bradyzoites differ extensively in the global gene expression pattern. Early realization of this fact came from stage-specific antigen detections in these two forms. It led to stage-specific cDNA library constructions and the identification of stage-specific genes (158-161). The highly used tachyzoite specific marker SAG1 and bradyzoite specific marker BAG1 were identified in such a manner (159, 161). Subsequently, the development of techniques like suppression subtraction hybridization (SSH) (162), microarray (163-171), comparative RNA-seq (172-176), and mass spectrometry-based comparative proteomics (177) have allowed comparison of the global gene expression patterns in tachyzoites and bradyzoites in high temporal resolution along the pathway of differentiation. Several major insights are gained from these "-omics" data that are also validated by other more targeted experiments. For example:

1) The transcriptome and proteome of bradyzoites remain dynamic for a long time even after the formation of bradyzoites in the mouse brain, and only become more static three months post-infection (173). This goes well with the observation that bradyzoites within tissue cysts are not completely dormant; instead, they continue to replicate and run other metabolic functions at a very low rate (169, 178, 179).

2) Several metabolic enzymes and some other proteins have isoforms in *T. gondii*, and they are differentially expressed in tachyzoites and bradyzoites. This was a well-known phenomenon that was discovered even before the use of -omics studies on

T. gondii (180, 181).

3) The cell cycle progression of tachyzoites shows two distinct stages of transcriptome, G1 phase and S/M phase (182). The transcriptome of a bradyzoite formation deficient mutant under stress condition has more similarity to S/M phase (169, 171). This supports the notion that during bradyzoite differentiation, a G2-like phase of the cell cycle is activated that is normally present in the eukaryotic cell cycle in between S and M phase but is not observed in the *T. gondii* cell cycle (183).

4) The tachyzoite transcriptome is differentially enriched for proteins in the TCA cycle and oxidative phosphorylation (174). This supports the currently held view that bradyzoites are more dependent on anaerobic glycolysis for energy production (149, 150).

1.5.1.3: Role of Epigenetic Regulators and Transcription Factors

The high number of differentially expressed genes between tachyzoite and bradyzoite suggests the presence of a global regulator of gene expression in this differentiation process. Epigenetic changes and transcription factors are obvious suspects that can bring such a high variation of global gene expression. A common epigenetic DNA modification in eukaryotes is cytosine methylation, catalyzed by DNA methyltransferases. DNA is more methylated in the bradyzoite stage compared to the tachyzoite stage. There are two DNA methyltransferases in *T. gondii*, both of which

are upregulated in the bradyzoite stage (184). On the other hand chemical inhibition or genetic knockout of epigenetic histone modifiers also affected the bradyzoite differentiation rate (171, 184-186).

The well-established role of AP2 TFs in *Plasmodium* differentiation motivated researchers to look for the role of their *T. gondii* homologs in bradyzoite differentiation. Knockout or overexpression of several AP2 TFs in *T. gondii* positively or negatively affected bradyzoite differentiation and cyst formation. However, none of these mutants could completely turn on or turn off the differentiation despite affecting the expression of several stage-specific genes (187-191). For example, AP2IX-9 expression peaks at two days post bradyzoite induction and declines thereafter, whereas AP2IV-3 expression rapidly increases also at day two and slowly keeps increasing or is maintained thereafter (189). AP2IX-9 is a putative transcriptional repressor that is shown to repress the expression of bradyzoite marker BAG1 (191). On the other hand, AP2IV-3 is a putative transcriptional activator that is demonstrated to activate BAG1 expression (189). In accordance with their role in BAG1 transcriptional regulation, AP2IX-9 and AP2IV-3 knockout mutants enhanced and reduced bradyzoite differentiation respectively (189). The overlap in the expression pattern of these two transcription factors exemplifies the highly dynamic nature of the early stages of bradyzoite differentiation. Indeed, parasites within a single vacuole or even a single parasite express markers of tachyzoites and bradyzoites together, during both bradyzoite-to-tachyzoite and tachyzoite-to-bradyzoite differentiation (192, 193).

The development of the CRISPR-Cas9 mediated forward genetic approach has been developed recently in *T. gondii* (194). With this approach, the bradyzoite formation phenotype was analyzed in ~200 knockout mutants (176). The tested gene set contained stage-specific genes, as well as known and putative transcription factors. Knockout of a single TF, BFD1, showed a bradyzoite formation deficiency phenotype. Furthermore, targeted disruption of BFD1 followed by single-cell RNA-seq based phenotypic characterization demonstrated that BFD1 is both necessary and sufficient for bradyzoite differentiation (176). This is the first identification of a master regulator of bradyzoite differentiation. How this TF itself is regulated in response to different classes of stress is still to be determined.

1.5.2: *T. gondii* Sexual Differentiation

Cats are the definitive host of *T. gondii*. They can get infected by tachyzoites, bradyzoite containing tissue cysts, and sporozoite containing oocysts. The preferred form for causing feline infections seems to be tissue cysts, as its prepatent period (time to shed oocyst in the feces) is 3 to 10 days, followed by tachyzoites (prepatent period >13 days), and oocysts (>18 days) (28). Life cycle progression in cat intestinal epithelial cells, determined by electron micrograph and described in only a few publications, is as follows:

The sporozoites from oocysts start infection, and replicate asexually via the tachyzoite replication cycle. Tachyzoites differentiate into bradyzoites, most likely due to immune system derived stress. The bradyzoites differentiate into five

morphologically distinct forms in a stepwise manner, potentially asexually replicating multiple times within each form. Finally, from a schizont-like form, merozoites appear that differentiate into male and female gamonts in the cat intestinal epithelium. Male gametes fertilize female gamonts to form zygotes, which are then differentiated into oocysts. Rupture of infected epithelial cells releases oocysts into the lumen that are ultimately excreted in the feces. Mitosis and meiosis produce two sporocysts, each containing four sporozoites (Figure 2). For a more detailed description of this life cycle with a description of each form, see reference (28). Until very recently, the bradyzoite to oocyst differentiation could only be observed in feline intestinal epithelial cells in vivo, which is a difficult experimental system to get access to (195). Therefore, molecular characterization of the intermediate stages is very limited (196, 197).

Despite the fact that cats are not a readily accessible experimental system, several comparative transcriptomics studies have been performed along this line of differentiation. Transcriptomes from three stages of oocyst maturation and merozoites were compared with in vitro tachyzoites and bradyzoites using microarray (198, 199). More recently, RNA-seq has been used to compare the transcriptome of merozoites, five separate timepoint samples along the sexual differentiation path from *T. gondii* infected cat intestinal epithelial cells, and tachyzoites (31, 200). Combining data from these experiments, stage-specific expression profile was determined for each of the stages and was used heavily to characterize a mutant parasite whose MORC gene can be conditionally knocked out (201). MORC is an ATPase protein that provides energy

for epigenetic modification of histone by histone deacetylase 3 (HDAC3) (201). Conditional knockdown of MORC, as well as chemical inhibition of HDAC3, silenced a large set of target genes (validated by Chip-seq), which are mostly enriched in gamonts and somewhat enriched in oocysts and bradyzoites (184, 201). It led to the conclusion that epigenetic histone modification by the HDAC-MORC complex suppresses parasite differentiation towards sexual replication. These are the only characterized global regulators of the *T. gondii* sexual differentiation pathway so far (201).

A long-standing question in the field of *T. gondii* was why the sexual cycle only takes place in the feline intestine. Recently, it was shown that the cat is the only mammal that lacks a specific fatty acid breakdown enzyme, which allows the buildup of linoleic acid in cats that triggers the sexual differentiation (202). Both linoleic acid supplementation in in vitro culture and inhibiting the degradation of linoleic acid in mice allowed the sexual cycle to commence both in vitro and in vivo in a mouse model (202). Even though the molecular detail of this signaling is still unknown, this discovery has made the experimental system of *T. gondii* sexual differentiation hugely accessible compared to the previous state (203). It could potentially lead to a better molecular characterization of *T. gondii* differentiation along the route of sexual stages.

1.6: *Cryptosporidium* Stage Differentiation

Cryptosporidium species are parasites of the vertebrate gastrointestinal tract (204). They can complete their life cycle within a single host. Many of the reported species can infect multiple hosts, both naturally and experimentally. For example, *C. hominis* is the major human pathogen of the genus and can also infect gnotobiotic piglets experimentally. *C. parvum* can infect both humans and cows naturally, and immunocompromised mouse models experimentally (205). Once considered as a coccidian, phylogenetic analysis now shows that this single genus constitutes a separate clade of Apicomplexa, which is phylogenetically more similar to gregarines than coccidians (206).

Oocysts are the infectious forms of the parasite that disseminate through the fecal oral route (Figure 3). Four sporozoites come out from an oocyst and infect intestinal epithelial cells (IECs). They form a parasitophorous vacuole (PV) within the host cell that is localized just under the plasma membrane, a peculiar localization that has often been described as intracellular but extracytoplasmic (207). Within the vacuole, *Cryptosporidium* transforms into trophozoites that develop into type I meronts containing six to eight type I merozoites, which come out and infect nearby IECs within a day post infection. After several rounds of this asexual replication, they differentiate into type II meronts containing type II merozoites, which in turn develop into gametocytes after infecting the IECs. Microgametocytes produce around 16 male gametes that come out and fertilize uninucleate female gametocytes residing in a host

cell. Gametocytes start to emerge 42 hours post *C. parvum* infection in vitro and they are the terminal form of *Cryptosporidium* differentiation in the human colorectal carcinoma cell line HCT-8, which is commonly used for in vitro cultivation. In vivo, fertilized zygotes differentiate into oocysts and, during their development, meiotic cell division within the PV forms four sporozoites. Two types of oocysts have been described for *Cryptosporidium*, thin-walled oocysts that autoinfect the host and thick-walled oocyst that are excreted into the environment (204, 208, 209).

Cryptosporidium infection is asynchronous, which makes it difficult to determine the number of asexual cycles before commitment to gametogenesis (204, 208, 209). The different stages are mainly described via electron microscopic observations (208, 210). Until very recently, there were no clear markers to differentiate different stages of the *Cryptosporidium* life cycle using light microscopy. The dogmatic view in the field is that sexual differentiation is an obligate step following several rounds of asexual replication. However, it is hard to determine if asexual cycles continue in parallel to sexual differentiation within the vertebrate host, because even if they are observed together, the asexual forms might result from subsequent rounds of infection from the autoinfective thin walled oocysts. Commonly used cell culture methods cannot support the life cycle completion in vitro, and cultures collapse after the majority of the parasites progress into gametocytes (211). Without molecular characterization, it is also difficult to determine if in vitro progression to gametocytes is predetermined or an induced event caused by in vitro culture conditions.

Recently, using a cleverly designed transgene cassette for *Cryptosporidium* that only supports fluorescent protein expression upon fertilization, it was shown that fertilization is blocked in the HCT8 cell culture system (212). One of the possible explanations for this observation can be that the gametocytes do not fully mature in the culture. Transcriptome comparison of *Cryptosporidium* at 48 hours and 96 hours post infection using RNA-seq reported that around 500 genes are differentially expressed between in vivo and in vitro conditions (213). However, the asynchronous nature of the parasite infection inhibited assigning differentially regulated genes to specific stages of the life cycle. 59 of the upregulated proteins in vivo are secreted proteins that include one of the major oocyst wall proteins and several other uncharacterized large glycoproteins that are potential oocyst wall components. In comparison, only six upregulated proteins in vitro are secreted proteins. While the proteins upregulated in vivo might be required for female gamete formation, they also might be expressed following fertilization. Therefore, macrogamete formation deficiency could not be completely verified in this study (213). A cleaner comparison of macrogamete development was performed by expressing a fluorescent protein under a female specific promoter and FAC sorting macrogametes from in vivo and in vitro conditions for RNA seq (212). This comparison did not suggest any major dysregulation of female gametocyte specific gene sets except that in terms of global gene expression in vivo females had comparatively more similarity to sporozoites compared to in vitro females (212). This observation can be explained by the fact that FAC sorting of the in vivo

population also isolated zygotes and immature oocysts, as they also fluoresce in this system (212). Potential microgamete development deficiency in vitro has not been tested robustly yet.

One of the recently developed stem cell-derived platforms that supports oocyst production in vitro utilizes differentiation of mouse intestinal stem cells at air-liquid interface (ALI) as a culture platform (214). The host cell differentiation is blocked in the absence of ALI condition, and consequently, *Cryptosporidium* growth is also significantly affected. This system is well suited to test the effect of host cell gene expression on *Cryptosporidium* differentiation. Comparison of ALI and non-ALI culture host cell transcriptomes suggest that several pathways are significantly differentially regulated between those two conditions. Specifically, the energy metabolism of the ALI culture significantly shifts from glycolytic to mitochondrial respiration (214). Taken together with the above-mentioned studies about macrogamete development, this suggests that the discontinuation of *Cryptosporidium* growth in different cancer cell lines may actually result from the differences in host cell characteristics rather than gametocyte development deficiency. However, the exact molecular mechanism of this phenomenon is still to be elucidated.

The transcriptome of sporozoites at varying in vitro culture time points has revealed that *Cryptosporidium* has a highly dynamic transcriptional regulation as it progresses through its life cycle, and there are numerous stage specific genes (213, 215, 216). A high throughput qRT-PCR based analysis has the highest temporal resolution

of transcripts so far (215). This study captured seven different time points ranging from 2 hours to 72 hpi in vitro. In combination, two RNA-seq studies also assayed the transcriptome of 2, 24, 48 and 96 hpi, as well as oocysts and sporozoites (212, 216). Despite differences in host cell lines, sampling times, methods used, and analyses modules, these three sets of data are fairly consistent and provide similar conclusions regarding several broad aspects of life cycle progression. For example, the gene expression pattern in intracellular stages is more diverse compared to sporozoites and oocysts, with a huge shift towards rapid transcriptional activities observed at early intracellular timepoints. Later time points in all the experiments showed enrichment of oocyst wall proteins and meiosis associated proteins. Importantly, the later time points contain a mixture of both male and female gametocytes in these experiments (213, 215, 216). RNA-seq mediated comparison between FAC sorted female parasites and asexual stages verified that both of the above-mentioned protein classes are enriched in female gametocytes (212). One interesting observation from both of the bulk RNA-seq experiments was that mRNA of one paralogue of two lactate dehydrogenase genes of *Cryptosporidium* is selectively highly expressed in the sporozoite population (213, 216). In a microarray experiment of oocysts, the same observation was reported (217). The potential functional significance of this observation is still to be realized.

The high temporal resolution of the qRT PCR dataset noted above makes it suitable for the exploration of co-expressed gene clusters. Indeed, in the original publication, they divided the genes into nine clusters based on their temporal

expression profiles (215). Subsequently, another study used the same dataset and, using an optimized parameter for clustering it described 200 different clusters of co-expressed genes (218). Several short DNA motifs were enriched in the promoter elements of several clusters (218). An in vitro AP2 TF DNA binding assay showed that some of the identified motifs are recognized by multiple *Cryptosporidium* AP2 TFs (219). For example, the motif "GCATGCA" was found to be overrepresented in 33 of 200 clusters (218). The DNA binding assay showed that slight variations of this motif are identified by four different AP2 TFs. Taken together, it suggests that AP2 TFs play important roles in stage-specific transcription of *Cryptosporidium* genes.

Several recent breakthroughs have made studying the differentiation of *Cryptosporidium* life cycle progression more amenable. The parasite is now genetically manipulatable using CRISPR-Cas9 technology (220), distinct phases of its life cycle can be identified by stage specific antibodies (221), completion of its life cycle is possible in vitro (214, 222-226) and stage specific assays are available (209). Together, these developments constitute a strong toolbox to progress our understanding of the *Cryptosporidium* life cycle beyond microscopic observation to molecular characterization.

1.7: *Eimeria* Stage Differentiation

Eimeria species are coccidian apicomplexans that can infect all classes of vertebrates and some invertebrates (227). Each species is considered to be specific for a single host, within which they complete their whole life cycle (227). Several *Eimeria* species are parasites of chickens that cause huge economic losses in the poultry industry. The most well studied species of them is *Eimeria tennela*.

Eimeria oocysts form sporocysts within them in the environment (Figure 4). Upon ingestion, they are released in the chicken gastrointestinal tract. Sporozoites come out of the sporocyst in the small intestine and infect the intestinal epithelial cells. After several rounds of asexual schizogony, where multiple merozoites from a schizont start another round of replication, gametes emerge, fertilization takes place, and subsequently, the zygotes differentiate into oocysts that are excreted in the feces (228). Based on the observations of morphologically different schizonts that emerge in a time-dependent manner after chicken infection, the dogmatic view is that *Eimeria* species are genetically programmed to replicate for a fixed number of asexual schizogony cycles before differentiating into gametocytes (228, 229). For example, upon *E. tennela* infection, first generation schizonts are observed at day 1 and 2 post-infection, the second generation of schizonts, which are larger and contain more nuclei than the first generation, are observed on day 3 and 4 post-infection, and third generation schizonts, which are smaller than both of the earlier schizont stages and contain up to 16 merozoites, are observed after 4 days of infection (228, 230). Merozoites from the

third stage schizont develop into gametocytes that start to emerge at day 5, which ultimately form the zygote. Oocysts are abundant in the feces on day 6 to 7 post infection (228, 230).

Several controlled experiments support the theory of preexisting genetic programs for the number of asexual replication cycles. For example, in a study testing this theory, merozoites were collected from chicken intestine 94 hpi (hours post-infection) and used to infect naïve chickens by direct intestinal injection (231). This inoculum is supposedly enriched for the second generation of merozoites and, in agreement with the theory, none of the earlier stage schizonts were observed in the naïve chicken. Third stage schizonts, gametocytes, and oocysts emerged at earlier time points compared to chickens infected with oocysts (231). Recently, RNA-seq and SSH techniques were used to determine the gene expression pattern of *E. necatrix* merozoites collected at 136 and 144 hpi. The two populations were considered the second and the third generation of merozoites respectively. A high number of genes were found to be differentially expressed and they were classified into second or third generation-specific genes (232, 233).

However, in vivo infection of *Eimeria* is asynchronous which makes it difficult to differentiate different stages of schizont based on size only (228, 229). Consequently, merozoite isolation at a certain time point post-infection would likely result in a diverse set of merozoites from different replication cycles. Additionally, instead of a replication number clock, there might be environmental signals that drive

the differentiation and as such, the parasites at different timepoints post infection might be at different stages of development/differentiation, irrespective of the number of schizogony cycles that they went through. Therefore, in the absence of specifically defined markers for each schizogony cycle and/or without genetic validations about the existence of distinct schizogony stages, the theory of a genetically determined replication number cannot be properly tested.

Attempts of attenuated vaccine development present another approach to test this phenomenon. It has been shown that repeated isolation of early oocysts and their selection by serial passage gives rise to less virulent precocious lines of *Eimeria* whose prepatent time decrease significantly from the parent parasites (234-237). Most commonly, fewer asexual replication cycles have been attributed to such shortened prepatent time of precocious lines (234, 237, 238). Searching for a potential genetic basis of the fewer asexual cycles that occur, one study crossed a precocious line with its parent and identified a genetic linkage in chromosome 2 associated with the shorter prepatent time phenotype (239). Recently, another study looked for differences in transcriptional activity of a precocious and its parent line and suggested potential differences exist in the expression of DNA replication related genes at merozoite stages (234). Heritable epigenetic changes can also cause such a phenomenon; however, epigenetic comparison between a parent and a precocious line has not been described yet.

To probe the commitment towards sexual differentiation in *Eimeria*, a study

used periodic acid-Schiff (PAS) staining in primary chicken kidney cell culture and reported that PAS-positive third generation schizonts form PAS-positive third generation merozoites that ultimately form PAS-positive macrogametocytes (240). The opposite was observed for microgametocyte development. This suggests that parasites are committed to forming either male or female gametocytes one replication cycle before the ultimate differentiation, similar to the *Plasmodium* parasites. However, this phenomenon has not been tested robustly with modern techniques.

Several studies have used comparative transcriptomics and proteomics analysis to understand the regulation of the life cycle progression of *Eimeria* (200, 232-234, 241-243). Analysis of the proteome of oocysts, in vitro sporulated sporozoites, and merozoites isolated from infected chickens suggested that merozoites express proteins involved in oxidative phosphorylation more abundantly, whereas glycolytic enzymes are expressed almost equally between sporozoites and merozoites (242). Another study used RNA-seq to determine differential expression of genes between sporozoites, asexual merozoites (collected 112 hpi) and gametocytes (gender independent, 134 and 144 hpi) (200). Genes that were upregulated in gametocytes contained enzymes for protein glycosylation and proteases, both of which classes were previously described as upregulated in gametocytes in independent studies (244, 245). Comparison of this dataset with *Plasmodium* sex-specific transcriptomic datasets showed that homologs of both male and female specific *Plasmodium* genes were upregulated in gametocytes in this dataset (200). Antisera raised against some of these proteins successfully

differentially identified male and female gametocytes (200), which could be very helpful for future differentiation studies.

Recently, a breakthrough publication in *E. tenella* described new gene knockout techniques in *Eimeria* using CRISPR-Cas9 mediated genetic manipulation techniques (246). The Cas9 protein was also successfully integrated into the genome, which creates a very amenable system for mutant library preparation (246). This study also demonstrated a successful knockout of 10 of the 33 putative AP2 TFs in the *E. tennela* genome, suggesting the others were essential (246). With this newly developed technique, long-standing questions in *Eimeria* life cycle progression can be addressed in a more comprehensive way. One still standing roadblock in the field is the absence of an easily manipulatable continuous culture system.

1.8: Stage Differentiation of Piroplasms

Piroplasms are a group of hematozoan apicomplexan parasites that infect blood cells of vertebrates for asexual replication and blood-sucking ticks for sexual replication (247). Phylogenetically, they are the closest relatives of *Plasmodium* among the parasites discussed in this review. *Babesia* and *Theileria* infect cattle while *Babesia* can also infect humans as a dead-end host (Figure 5). Hard ticks of the family Ixodidae are their definitive host who takes up the mature gametocytes of these parasites residing in RBCs of mammalian hosts (248). In the tick midgut, gametogenesis and fertilization takes place, zygotes are formed in the midgut cells,

followed by meiosis to produce kinetes. Kinetes migrate to the salivary gland of the tick via hemolymph, and differentiate into sporoblasts that contain sporozoites, which are the vertebrate infective form. *Babesia* sporozoites directly infect RBCs, while *Theileria* sporozoites first infect leukocytes. *Theileria* merozoites released from leukocytes then infect RBCs. The asexual replication cycle and occasional gametocytogenesis occur within RBCs, ultimately forming mature gametocytes. Even though there are several steps in the piroplasm life cycle where differentiation occurs, our knowledge about molecular mechanisms governing these steps is extremely limited (248).

1.8.1: *Theileria*

Most of the pathogenic *Theileria* species can cause an oncogenic transformation of the leukocytes that they infect (249). They replicate via schizogony inside the parasitophorous vacuole within a transformed cell, and when the host cell is mitotically divided, they are distributed into daughter cells (250). Importantly, this transformation is reversible. Typically, *Theileria* are cultivated in vitro in transformed leukocytes (249). Different aspects of this transformation process have been studied relatively more extensively than progression of the parasite beyond this stage of the life cycle (251). They progress to the RBC stage by producing merozoites that egress from the leukocytes. The observation that the number of nuclei in the schizont increases before merozoite formation led to the theory that a high ratio of DNA compared to protein in the parasitophorous vacuole signals merozoite differentiation (252). Inhibition of DNA

synthesis using a cell cycle progression inhibitor induced early differentiation, whereas a mitochondrial protein synthesis inhibitor prolonged the time before differentiation (253). This led to a more refined theory that when specific protein factors in the parasitophorous vacuole reach a threshold, it induces commitment and the higher ratio of DNA in the earlier theory on commitment is just a facilitator for higher transcriptional activity (i.e. higher number of genomic copy allows higher transcription of a specific factor) in the multiple nuclei containing parasitophorous vacuole (253). It was subsequently shown that a protein overexpressed during differentiation is transcriptionally regulated by putative transcription factors that are also enriched during differentiation (254). This supported the notion that reaching the threshold of specific factors drives differentiation (254). Increasing culture temperature is the most common way of inducing merozoite differentiation in *Theileria* (252-255). It was shown that the protein synthesis rate of the parasite increases at the higher temperature (253). Finally, stage-specific overexpression of 3 genes was verified by northern blotting (255).

All these pre-omics era studies suggest that there is a stage specific expression of genes that drives differentiation. Indeed, transcriptomic and proteomics studies confirm that there are stage specific expression profiles of genes in different stages of the *Theileria* life cycle, just like all the other Apicomplexa (256-258). However, even though there are markers of different stages, wet lab experimental identification of a master regulatory element is still missing. In silico analyses have shown that *Theileria* contains several AP2 TFs, and expression profiles of several of those AP2 TFs are also

stage specific (259, 260). The genetic knockout system is still not developed for *Theileria*, however, so potential experimental validation approaches are limited at this point (261).

1.8.2: *Babesia*

Several species of *Babesia* can be continuously cultured in-vitro within RBCs during their asexual replication cycle (262). No schizogonic replication has been documented for *Babesia* yet (248). In the mammalian blood, some parasites differentiate into gametocytes, which are activated in the tick midgut to form gametes (248). Induction of gametocytogenesis, as well as gametogenesis, can be achieved in in vitro blood culture of *Babesia* (263-265). Stressing the parasites with anti-*Babesia* drugs induces gametocytogenesis, whereas induction with xanthurenic acid or tick extract induces both gametocytogenesis and gametogenesis (265, 266). There are three validated marker genes of gametocytogenesis, two of them are expressed in kenetes also (267, 268).

Hap2 is a well documented male gamete specific protein in different systems, and it is functionally important for gamete fertilization (269). Reducing Hap2 activity, either by functional inhibition using antibodies or by genetic knockout, inhibits zygote formation, suggesting a similar role for Hap2 in *Babesia* as seen in the other systems (270, 271). A putative methyltransferase is only expressed in tick stages (268). In addition to these markers, there are other potential stage specific proteins in *Babesia* that are identified through proteomics based analysis (272-274).

Global regulators of differentiation have yet to be identified for *Babesia*, and none of the AP2 TFs have been studied for differentiation in a targeted way yet (260). As a gene knockout system in *Babesia* is available (271, 275), knockout mutant derived validation of a master regulator is possible, and the already established stage specific markers would greatly help in that process.

1.9: Similarities and Differences in Stage Differentiation Between Apicomplexan Parasites

During almost the entirety of their life cycle, all the discussed apicomplexan parasites have a haploid nucleus. Zygotes are the only diploid stage, and in most cases, they are very short-lived because of the subsequent meiotic division. Whether there are common underlying mechanisms of initiation of meiosis in response to the diploid state of the nucleus is not known.

Sexual differentiation of the Apicomplexa starts with gametocytogenesis, which gives rise to male and female gametocytes. The two gametocytes develop with highly divergent gene expression patterns, but they normally emerge together during the course of infection. This suggests a two-step process of gametocytogenesis induction. First, a gender independent commitment step, and subsequently, a gender-specific differentiation pathway. This has been well characterized in *Plasmodium* where Δap2-g mutants do not form any gametocytes whereas Δap2-fg are only deficient in female gametocyte maturation. A sexually committed schizont stage is observed in

Plasmodium within which the two above mentioned steps likely take place. Hints of the same phenomenon have been also observed in *Eimeria*, where the schizonts in the last cycle are morphologically distinct from the earlier cycles, and in *Cryptosporidium*, where a type II meront is observed before the emergence of gametocytes. Whether these two stages are analogous to the sexually committed schizont of *Plasmodium* has not yet been tested with molecular techniques.

The time interval between commitment to gametocyte formation and the ultimate release of gametocytes differs between different apicomplexan species. For hematozoans, mature gametocytes of both sexes need to come out from the host cell at the same time in the vector midgut. In the case of Coccidians and *Cryptosporidium*, only the male gametes egress from the host cells, after which they fertilize mature female gametocytes residing in other host cells. In any case, gametocyte maturation of both sexes must be synched in time for successful fertilization.

Gametocyte development pathways seem to be well conserved within apicomplexans, as multiple transcriptomics and proteomics studies have shown that homologous proteins are expressed in a sex-specific manner for all the apicomplexans. For example, meiosis-related genes are expressed during the maturation of female gametocytes in different parasites, presumably so that the proteins for meiotic division are readily available soon after the fertilization. On the other hand, expression of HAP2, a well-characterized gamete fusion protein, is restricted to male gametocytes in different apicomplexans.

Commitment to the next step of differentiation occurs before the ultimate stage conversion in almost all of the differentiation processes. The only exception seems to be the tachyzoite to bradyzoite differentiation, which is more dynamic. However, this particular differentiation is also unique in the sense that it is reversible. Even including bradyzoite differentiation, identified global regulators of differentiation in different apicomplexans are highly common between species. The family of AP2 TFs plays key roles in all the differentiation processes. Post-translational regulation is also commonly observed in many differentiation processes. For example, inhibition of translation initiation by phosphorylation of eIF2α is observed for *Plasmodium* sporozoite to liver stage conversion, and in tachyzoite to bradyzoite conversion. However, this conservation can be artificially biased, as the selection of global regulators for in-depth studies is often guided by what is already known in other apicomplexans. Indeed, when a relatively unbiased mutant library screen was used to identify key regulators for bradyzoite differentiation, it identified a myb domain containing TF, which was never implicated in apicomplexan differentiation before (176).

Stage specific alteration in energy metabolism is also a well conserved feature of apicomplexan differentiation. Some stages are more dependent on anaerobic glycolysis for energy production, whereas other stages are more reliant on oxidative phosphorylation. The role of epigenetic modification of chromatin structure in differentiation is starting to emerge in apicomplexans, and will likely be recognized to play a role in many apicomplexans after further study.

1.10: Future perspectives

Several technological advances in recent years have made studies of apicomplexan differentiation more accessible. Two techniques have potential high values in understanding differentiation across multiple apicomplexan species—namely, single cell RNA-seq and CRISPR-Cas9 mediated mutant library preparation.

It is often difficult to differentiate different stages of the apicomplexan life cycle due to their asynchronous growth and lack of robust stage-specific markers. In addition, the lack of an easily manipulatable in vitro culture system hinders studies of different apicomplexans. Theoretically, single cell transcriptomics can overcome all these challenges. Great examples of this are the two single cell transcriptional atlases of *Plasmodium* and *T. gondii* (129, 172). From both of these studies, it is apparent that single cell transcriptomes from parasites at different stages of their life cycle cluster with unguided algorithms in a stage specific manner. Deductive reasoning can be applied to such datasets from our knowledge on well-studied apicomplexans to determine the identity of each cluster. As transcriptomes are generated from single cells rather than a population, they are theoretically more suitable for identifying sets of co-expressed genes. Additionally, it is almost the ultimate phenotypic characterization tool when the phenotype is the differentiation capability .

The second technique, CRISPR-Cas9 mediated mutant library preparation, is relatively restricted to parasites with already established robust transfection and genetic manipulation systems. It is already available for *Plasmodium* and *T. gondii*, and also

probably for *Eimeria* (58, 194, 246). CRISPR-Cas9 mediated library generation allows preparation of an unbiased mutant library, as well as relatively straightforward determination of library composition in various conditions. The utility of this technique has already been seen for *Plasmodium* liver stage metabolic map construction, as well as in the identification of a master regulator of *T. gondii* bradyzoite differentiation (58, 176).

There are still a lot of unanswered questions in apicomplexan differentiation. With the techniques that are now available together with unprecedented access to a wealth of "-omics" data, a lot of those questions are ready to be answered.

1.11: Summary of our approach

We have applied a systemic chemical genomics approach to understand the regulation of *C. parvum* differentiation. Using a stage specific differentiation assay, we screened a library of more than 10,000 compounds and identified compounds that specifically inhibit the sexual differentiation or maturation of *C. parvum*. The transcriptomic responses of the parasites to these sexual stage specific inhibitors were then determined using mRNA-seq. To our knowledge, this is the first time a chemical genomic approach has been used to study the process of differentiation in apicomplexans. Our data greatly complement the already published transcriptomics data available on *Cryptosporidium* life cycle progression. By robustly comparing our dataset with already published datasets of *Cryptosporidium* differentiation and other

apicomplexans, as well as analyzing the already known mode of actions of several sexual stage specific inhibitors, we have put forward several hypotheses regarding potential master regulatory elements and metabolic alteration of *Cryptosporidium* in the course of sexual differentiation. Our data highlight the potential commonalities of *Cryptosporidium* and other apicomplexans in driving differentiation, as well as suggesting some unique aspects of *Cryptosporidum* biology.

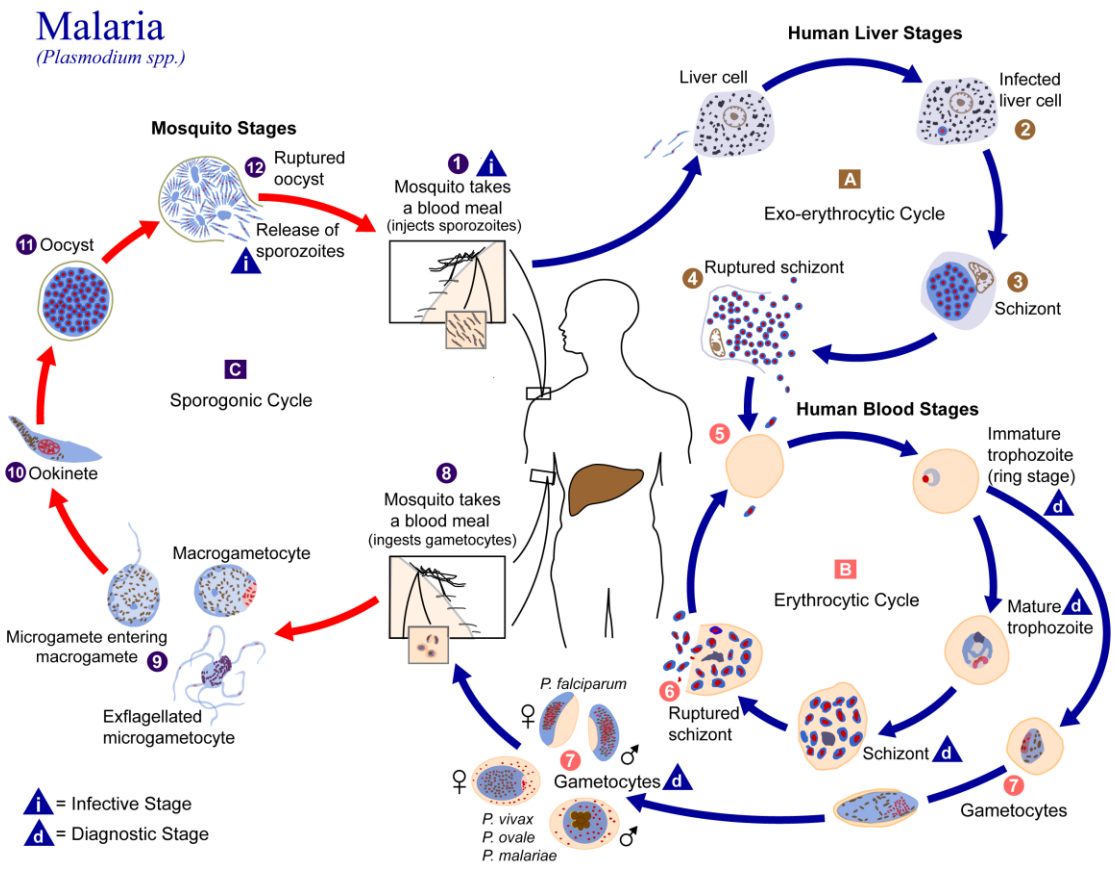

Figure 1: *Plasmodium* life cycle.

See section 1.4 for detailed description. The illustration is a public domain image by "CDC - DPDx/ Alexander J. da Silva, PhD; Melanie Moser" and downloaded from Public Health Image Library (PHIL)

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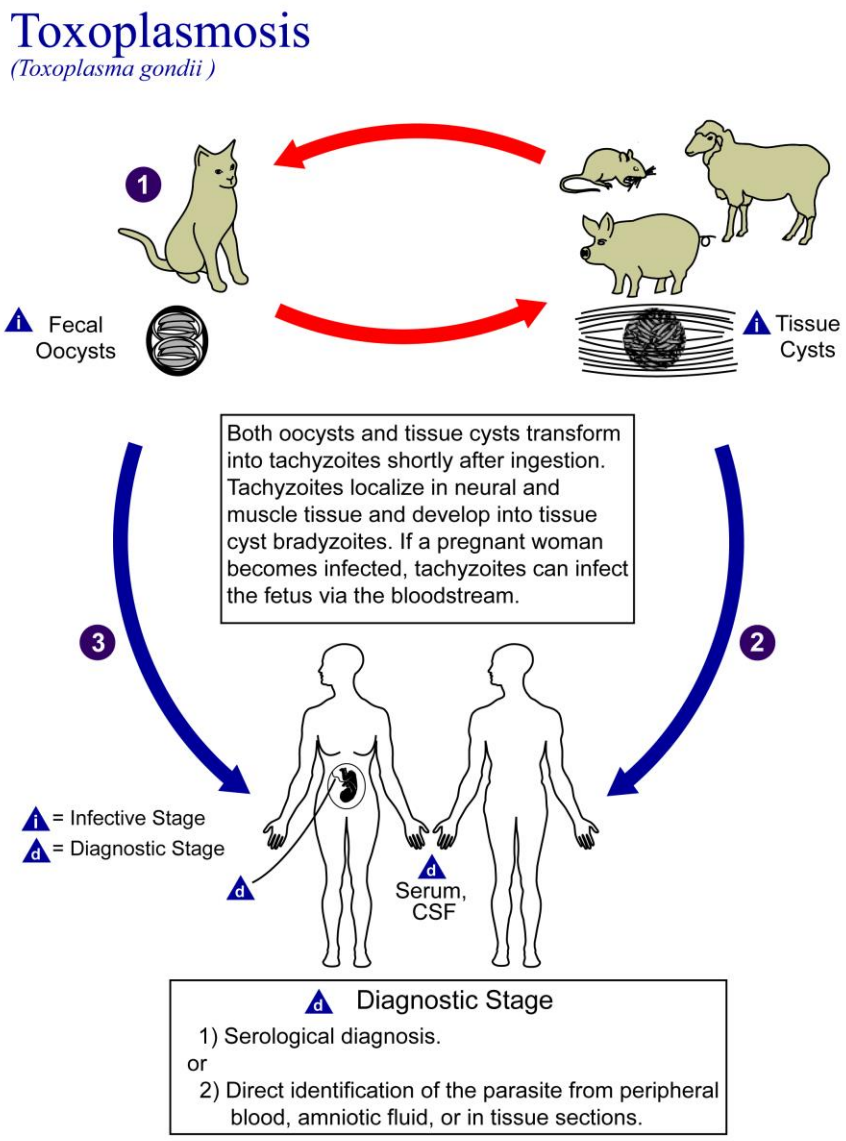

Figure 2: *Toxoplasma gondii* life cycle.

See section 1.5 for detailed description. The illustration is a public domain image by "CDC - DPDx/ Alexander J. da Silva, PhD; Melanie Moser" and downloaded from Public Health Image Library (PHIL).

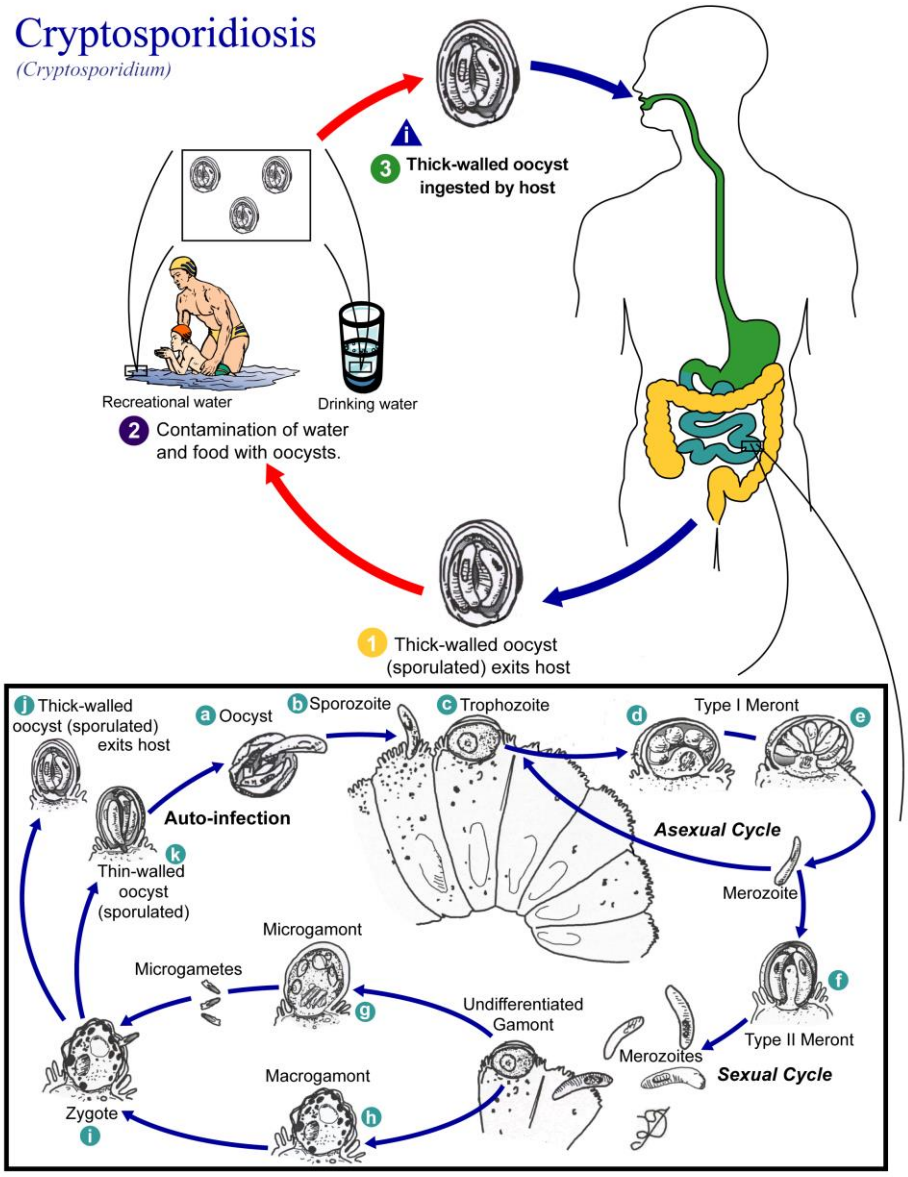

Figure 3: *Cryptosporidium* life cycle.

See section 1.6 for detailed description. The illustration is a public domain image by "CDC - DPDx/ Alexander J. da Silva, PhD; Melanie Moser" and downloaded from Public Health Image Library (PHIL).

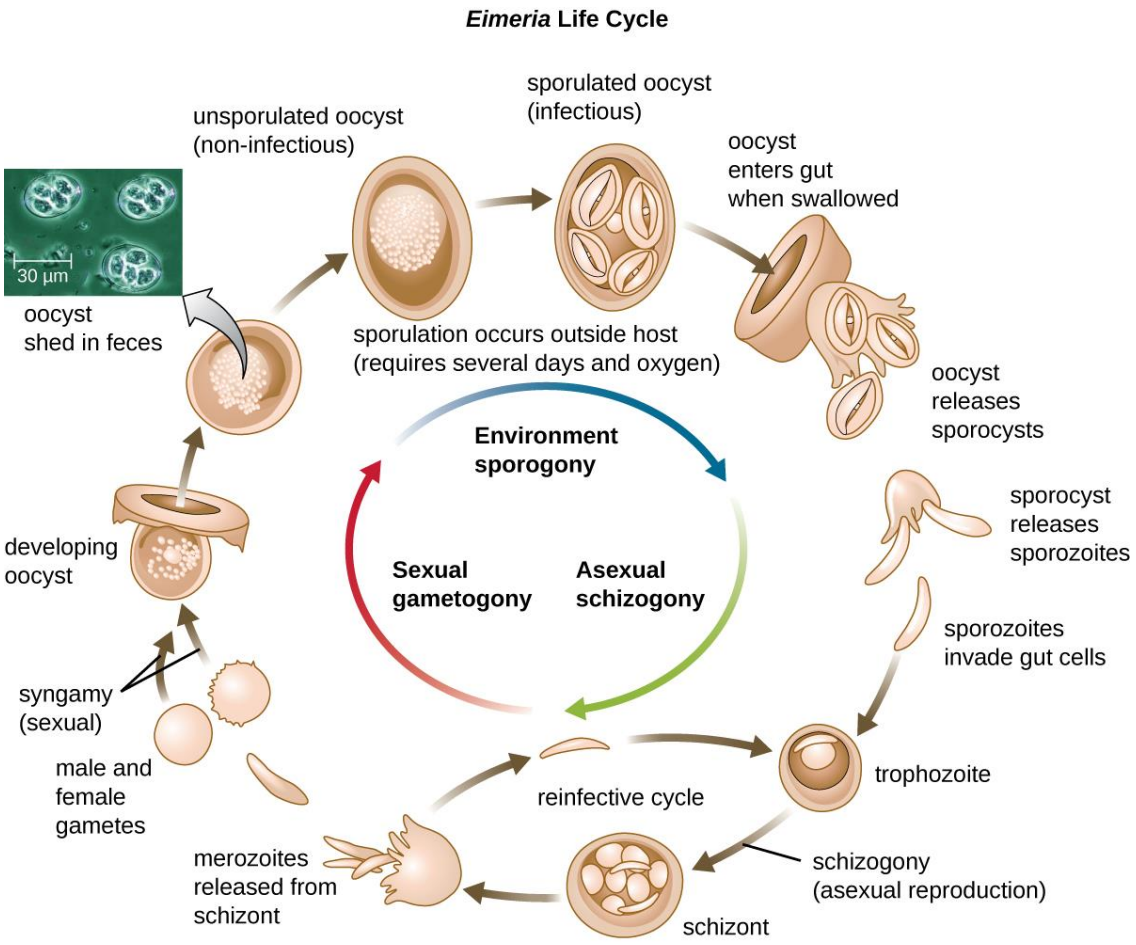

Figure 4: *Eimeria* life cycle.

See section 1.7 for detailed description. The illustration is licensed under "CNX OpenStax / CC BY (https://creativecommons.org/licenses/by/4.0)" and is downloaded from https://commons.wikimedia.org.

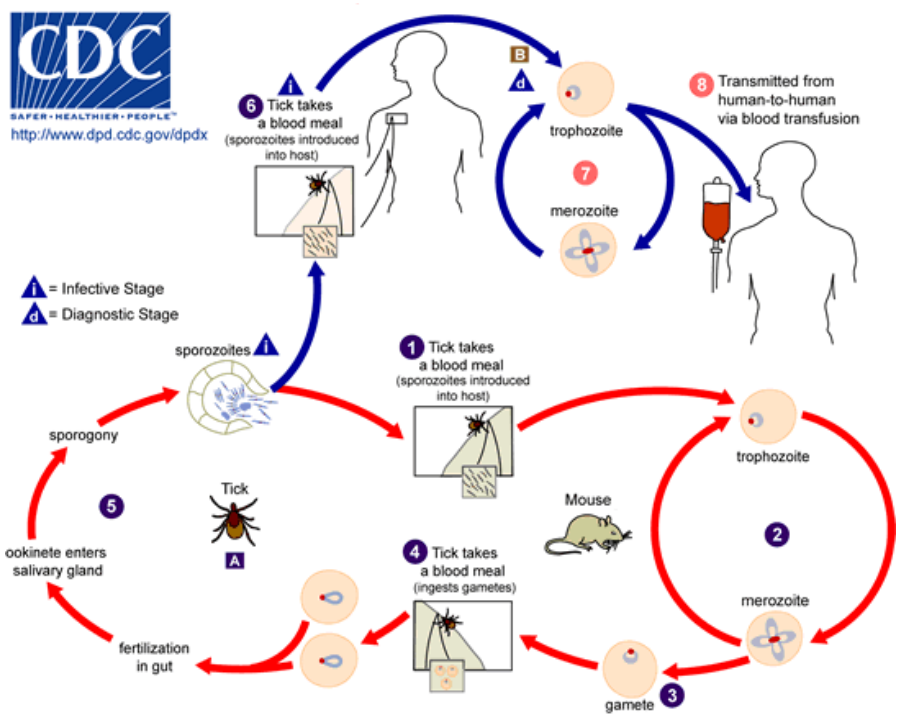

Figure 5: *Babesia* life cycle.

See section 1.8 for detailed description. The illustration is a public domain image and is downloaded from cdc.gov.

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CHAPTER 2: A CHEMICAL GENETICS APPROACH TO UNDERSTAND THE REGULATION OF CRYPTOSPORIDIUM SEXUAL DIFFERENTIATION

2.1: Abstract

Cryptosporidium is an intracellular protozoan parasite that is a leading cause of childhood diarrhea in Africa and Southeast Asia. It is a member of eukaryotic phylum Apicomplexa, a group of intracellular parasites that replicate both sexually and asexually in the course of their life cycle. Sexual differentiation of *C. parvum* is required for host to host transmission of the parasite as well as for autoinfection of the same host. In this study, we have optimized and utilized an in vitro high content imaging assay to test compounds for activity against the sexual and asexual stages of the parasite separately. The tested compounds comprise the ReFRAME library, a drug repurposing library composed of ~12,000 compounds for which extensive prior knowledge exists including their putative targets and modes of action. Inhibitors of the host cell's electron transport chain and purine nucleotide biosynthesis disproportionately affected the sexual differentiation of *C. parvum*. To identify the stage specific genes and pathways as well as the potential key regulators of the sexual differentiation process, we used RNA-seq following treatments with nine differentiation inhibitor and found that a number of parasite genes were dysregulated with multiple compound treatments. Ribosomal protein genes were upregulated with differentiation inhibitor treatments, which suggests that global repression of protein

translation is associated with *C. parvum* sexual differentiation. Analysis of publicly available RNA-seq datasets of *C. parvum* and other apicomplexan parasites indicates that this is a common mode of regulating apicomplexan stage differentiation. Our data also implicate several apicomplexan AP2 transcription factors as key regulators of *C. parvum* sexual differentiation.

2.2: Introduction

Cryptosporidium species are one of the most significant etiologic agents of childhood diarrhea in developing countries (1). There is an urgent need to develop novel therapeutics, as current treatment options are inadequate for treating malnourished children and immunodeficient patients, the two populations most vulnerable to cryptosporidiosis (2). The parasite is a member of the eukaryotic phylum Apicomplexa, which is comprised of intracellular protozoan parasites. Malaria and toxoplasmosis are two other major human diseases caused by apicomplexan parasites.

Cryptosporidium transmits through the fecal-oral route in the form of environmentally resistant oocysts. In the mammalian gut, the oocysts excyst to release sporozoites that infect the intestinal epithelial cells (IECs). Initially, it replicates asexually where intracellular trophozoites develop into type I meronts that release up to 8 merozoites that can repeat this asexual cycle. After several rounds of asexual replication, parasites differentiate into type II meronts (containing 4 merozoites instead of 8), and merozoites from type II meronts differentiate into gamonts upon infecting

IECs. Gamonts are of two types: uninucleated macrogamonts (i.e. female gamonts), and multinucleated microgamonts (i.e. male gamonts). Male gamonts fertilize female gamonts by infecting a macrogamont-harboring IEC. Meiotic cell division in the fertilized gamont produces 4 sporozoites that are subsequently enclosed by an oocyst wall and released from the host cell. Oocysts either excyst within the same host to cause autoinfection or are released into the environment with feces (3).

In the laboratory setting, oocysts can be excysted by treating with sodium taurocholate, and the sporozoites can infect numerous cell lines (4, 5). A widely used in vitro model of *Cryptosporidium* infection involves infecting the human colorectal carcinoma cell line HCT8 with the human pathogen *C. parvum*. In this system, after initial asexual replication, the gamonts start to emerge at 36 hours post-infection (hpi) and they are the predominant parasitic form at 72 hpi (6-8). However, fertilization does not take place within this system, and parasite replication therefore halts after sexual differentiation (7). Parasitophorous vacuoles of all life cycle stages of *C. parvum* can be stained with *Vicia villosa* lectin (VVL). Previously, we have also reported a marker protein for female gamonts, DNA meiotic recombinase 1 (DMC1), that can be visualized by immunostaining (6). As both male and female gamonts emerge within a similar temporal window post-infection, we used DMC1 expression as a surrogate for *C. parvum* sexual differentiation and developed an assay to test the activity of drug-like small molecules against sexual stages of the parasite. Several anticryptosporidial compounds, initially identified by screening using *Vicia villosa* lectin staining, showed

a diverse range of activity against the sexual stage of the parasite (6).

Like *C. parvum*, all the apicomplexan parasites cyclically differentiate into distinct forms in the course of their complete life cycle. Each of the stages has its own signature global gene expression pattern (7, 9-12). Molecular understanding of stage differentiation could provide an opportunity to develop novel control strategies against human pathogens. *Plasmodium* species, the causative agents of malaria, first replicate in hepatocytes upon infecting humans, then differentiate to infect red blood cells, and finally differentiate into gametocytes that are taken up by mosquito vectors where sexual reproduction takes place. Transmission blocking strategies for malaria therefore include inhibiting sexual differentiation of *Plasmodium*, whereas inhibiting the liver stage development or its differentiation into the blood-stage would be useful strategies for malaria prophylaxis (13, 14). Fast replicating *Toxoplasma gondii* tachyzoites differentiate into slowly replicating bradyzoites and form tissue cysts in response to immune activation in mammalian hosts that can be reactivated upon immune suppression. Intervening with this reversible differentiation could lead to better management of toxoplasmosis (15). *C. parvum* is different from *Plasmodium* and *Toxoplasma* in the sense that it can complete its whole life cycle within a single mammalian host where its sexual differentiation is believed to be an obligatory step (as opposed to the facultative gametocytogenesis observed for *P. falciparum* where only a small subset of total parasites actually differentiate into gametocytes). Therefore, inhibiting *Cryptosporidium* sexual differentiation may be a good therapeutic strategy

(8).

Apicomplexan stage differentiation has been studied with several complementary experimental approaches. Comparative "-omics" analyses between life cycle stages are useful in identifying stage-specific genes and pathways (16-19). Genetic manipulations, either through forward genetic screens or through targeted manipulation of key regulators of differentiation, have also been used to study the regulation of apicomplexan life cycle progression (20-24). Finally, chemical biology approaches have identified numerous tool compounds to study specific aspects of apicomplexan biology (25).

In this study, we have used a chemical genetics approach in combination with mRNA-seq to better understand the regulation of *C. parvum* sexual differentiation. We optimized a high content microscopy screening assay based on DMC1 expression, and screened the ReFRAME (Repurposing, Focused Rescue, and Accelerated Medchem) chemical library (26) in order to identify compounds that affect *C. parvum* sexual differentiation. The ReFRAME library is a library of ~12,000 compounds with high potential for drug repurposing, and, since the mechanism of action of many ReFRAME library compounds is known, we reasoned that this approach would enable us to gain mechanistic insights into *C. parvum* sexual differentiation. We then followed screening hit confirmation with mRNA-seq experiments in the presence or absence of selected differentiation inhibitors. Our results indicate that transcriptional repression of proteins involved in ribosome biogenesis is necessary for *C. parvum* sexual differentiation.

2.3: Results

2.3.1: Optimizing the Identification of Sexually Differentiated *C. parvum*

Immunostaining DMC1 protein was used to identify sexually differentiated *C. parvum*. In order to maximize the sexual differentiation of the parasite and optimize the identification of DMC1 expressing ($DMC1^+$) parasites in the HCT8 cell infection model, we made several modifications to our standard culture, staining, and image analysis methods. Key changes included supplementing the media with 1% serum instead of 10% serum, addition of the antifungal compound Amphotericin B, using 1% BSA instead of 4% BSA as blocking solution during immunostaining, and creating an image mask for all the parasites in the culture (identified by VVL staining) before locating high DMC1 signal during automated parasite counting. In the end, we managed to obtain a good signal-to-noise ratio for the $DMC1^+$ parasites at 72 hours post infection (hpi) (Figure 1A). The Z' score, an indicator of assay quality (27), was consistently > 0.7 for the identification of $DMC1^+$ parasites when using "no primary antibody" staining as our negative control.

The $DMC1^+$ parasite ratio detected with this method at 48 hpi was almost zero when using a 0.45 NA objective as is needed for screening with our automated microscope (Figure 1B). We note that other studies reported a higher proportion of female gamonts at 48 hpi when detected using other methods (7, 8), and, in our previous study, we also detected a higher proportion of $DMC1^+$ parasites at 48hpi when visualized through an objective with more resolving power (6). The ratio of sexual

stage parasites to total parasites that we detected with the current method at 72 hpi was very similar to all the prior reports (6-8). This indicated that DMC1 needs to be highly expressed to be detected by our screening method, suggesting that it detects only relatively mature female gamonts.

For compound library screening, we devised a strategy in which we tested each compound in three separate conditions and considered four separate readouts (two asexual growth readouts and two sexual stage specific) (Figure 1C). The asexual stage specific readouts were calculated by counting the total number of parasites at 48 and 72 hpi following the addition of compounds at 3 hpi. The number of parasites was expressed as a ratio to the number of host cell nuclei to control for host cell cytotoxicity, and the effect of compounds on parasite growth was calculated as percent inhibition relative to vehicle control-treated wells on each assay plate. The sexual stage specific conditions and readouts were as follows: 1) addition of compound at 3 hpi and determination of the ratio of $DMC1^+$ parasites at 72 hpi; and 2) addition of compound at 48 hpi (when very few $DMC1^+$ parasites are detectable) with the determination of the ratio of $DMC1^+$ parasites at 72 hpi. We reasoned that compound addition at 3 hpi would allow us to identify compounds that inhibit sexual differentiation but minimally impact asexual replication. Note that this readout identified compounds that affect the commitment to differentiate with minimal effect on asexual growth, but for compounds that inhibit asexual growth, it yielded little useful information about differentiation due to low parasite numbers. The second sexual stage specific readout with compound

addition at 48 hpi addressed this, since it identified compounds that inhibit the development of female gamonts following the commitment to differentiate independently of the compound's effect on asexual growth.

2.3.2: Screening and Validation of the ReFRAME Library

Using the combined stage specific assay strategy, we screened the ReFRAME compound library at 2 µM concentration. We had multiple DMSO control wells in each plate as well as two wells of known inhibitor control nitazoxanide. Each compound's activity for each readout was determined by normalizing the parasite counts with that of the DMSO wells from the same plate. In the end, we determined the average activity and the standard deviation for all the compounds in the library in each readout and compounds showing activity two standard deviations above or below the library mean were denoted as hits in each readout (Figure 1D and 1E). For initial follow up on compounds meeting this hit definition for at least two of the four screening readouts, we used resupplied compounds to conduct eight-point dose response assays, ranging from 50 nM to 10 µM for each assay readout (a total of 311 compounds).

The compounds roughly fit into seven different categories based on the dose response curves (Figure 2A). 42 compounds showed inhibitory activity in all four readouts with similar potency. These were denoted as pan-inhibitors, and were felt to be the most straightforward drug-like compounds for the potential treatment of cryptosporidiosis (Figure 2B). 70 compounds showed no dose-dependent activity in

any of the readouts and were considered false positives from the screening (Figure 2C). All the other compounds were differentially potent among the readouts and together they constitute a diverse set of tool compounds for studying the *C. parvum* life cycle. The most common theme (accounting for 102 compounds) was similar potency for inhibition of all readouts except for inhibition of $DMC1^{+}$ parasites upon addition at 48 hpi (Sexual Readout 1 in Figure 1C). These compounds were either completely inactive in this readout (Figure 2D) or showed dose dependent activity that failed to reach complete inhibition at the highest tested concentration (Figure 2E). We categorized these compounds as asexual inhibitors. On the other hand, 23 compounds were highly potent against the sexual stage when added at 3 hpi, but had little effect in the other conditions/readouts (Figure 2F). These compounds were categorized as differentiation inhibitors and were the predominant focus of our follow up experiments. A further 9 compounds showed a narrow but discernable specificity also in the sexual differentiation readout (Figure 2G). 19 compounds induced sexual differentiation (Figure 2H) or sexual development (Figure 2I) or both (Figure 2J) at concentrations that were not inhibitory to asexual stages in a dose dependent manner. We did not classify 46 compounds in any of the above-mentioned categories mainly because they were not tested in all conditions or their dose response curves seemed to be in the exponential phase and did not reach the above 80% inhibition threshold at the highest concentration tested (Figure 2K). Interestingly, a particular differentiation inducer, the Endothelin A receptor agonist S-1255, highly induced the expression of DMC1 at 48

hpi, which was unique among all the compounds that we tested (Figure 2L). Supplementary Table 1 contains the SMILES structures of all compounds tested in follow up, their putative targets and how they were categorized. Supplementary Figure 1 contains their dose response curves for each assay readout.

2.3.3: Several Differentiation Inhibitors Share Common Modes of Action and Activity Kinetics

The compounds categorized as differentiation inhibitors completely inhibited the emergence of $DMC1^+$ parasites in a dose dependent manner. However, they did not completely inhibit asexual parasite growth at any concentration tested (Figure 2F). All 23 of them had minimal or no effect on DMC1 expression when added at 48 hpi, suggesting that they worked by inhibiting early steps in the differentiation pathway. Interestingly, four of them were mitochondrial electron transport chain inhibitors (Figure 3A), five of them were inhibitors of nucleotide biosynthesis (Figure 3A, 3B), and two of them were fumagillin analogs that covalently inhibit methionine aminopeptidase 2 (Figure 3A). Interestingly, the confirmed asexual stage inhibitors also included compounds that inhibit nucleotide biosynthesis, but the putative targets of differentiation inhibitors were exclusively involved in the synthesis of purine nucleotides (Figure 3B).

We decided to further probe the mechanisms underlying *C. parvum* sexual differentiation using several of these tool compounds. We purchased seven differentiation inhibitors from independent sources and two were resupplied from the

ReFRAME library (Figure 3A). These nine compounds were selected because they showed a highly selective inhibitory activity against sexual differentiation for a wide range of concentration. We first tested a broader range of concentrations for each for the condition 2 readouts, which reconfirmed the initial dose dependent characterization for all nine compounds (Figure 3C).

Previous experiments looking at *C. parvum* life cycle stages at different time points post infection suggest that sexual differentiation takes place between 36 and 48 hpi (7, 8). This view is consistent with our observation that all the differentiation inhibitors were highly efficacious in inhibiting sexual differentiation when added 3 hpi, but ineffective to do so when added 48hpi. While this roughly indicates that they need to be added before the differentiation takes place, we wanted to test this phenomenon with a higher temporal resolution. To probe if there is a temporal window when the differentiation inhibitors need to be present to exert their effect, we added the nine retested differentiation inhibitors at 3, 12, 24, 34 or 48 hpi, and determined the ratio of $DMC1^+$ parasites at 72 hpi (Figure 4A). Six compounds showed highly efficacious dose dependent activity when added up to 34 hpi. On the other hand, three compounds (i.e. pralatrexate, mycophenolate, and TVB-2640) lost that activity if added at or after 24 hpi (Figure 4B), suggesting that prolonged exposure was required and/or that they affect early stages of differentiation such as commitment. Nitazoxanide, which was classified as a pan-inhibitor, had the same effect regardless of the timing of addition. This was consistent with the likelihood that pan inhibitors have a general toxic effect on

C. parvum growth that is independent of the stage of the parasite's life cycle.

2.3.4: mRNA-seq Analysis of Compound Effects on Gene Expression

To gain insights into the genetic regulation of *Cryptosporidium* sexual differentiation, we examined the effects of selected differentiation inhibitors on parasite and host gene expression using mRNA-seq and the *C. parvum* HCT8 cell culture system. A suitable time point was first determined by conducting mRNA-seq in the absence of differentiation inhibitors at 18, 36, 48, and 72 hpi. We aligned the sequencing reads with both human and *C. parvum* genomes using HISAT2 (28), created separate subsets of alignment files uniquely aligned to the parasite or host genome, counted the number of alignments per gene using HTseq (29), and finally performed differential gene expression analysis using DEseq2 (30). Genes with ≥ 2-fold expression difference at a 10% false discovery rate (FDR) were considered as differentially regulated. Overall, very few parasite genes were differentially regulated between the 18 and 36 hpi samples, but a large number of genes were differentially regulated between the 36 and 48 hpi samples and the 48 and 72 hpi samples. On the other hand, a relatively small number of host genes were differentially expressed (Figure 5A). Given these data, we selected the 48 hpi timepoint for the transcriptomic analysis of the differentiation inhibitor treatments in subsequent experiments. Each compound was used at a dose that was previously found to be highly selective for sexual differentiation (see Figure 3C), and mRNA-seq libraries were prepared at 48 hpi after each of the compounds or vehicle were added at 3 hpi (Figure 5B)(2 biological

replicates each, except for pralatrexate (n=1) for which library preparation failed for one replicate).)

Compound treatment affected both host and parasite gene expression (Figure 5B). The two methionine aminopeptidase2 inhibitors, AGM-1470 and Beloranib hemioxalate, very selectively dysregulated parasite gene expression, whereas the inhibitors of oxidative phosphorylation and mubritinib dysregulated a high number of host genes. We also performed the differentiatial gene expression analysis using the the combined expression count files of the host and the parasite genes together, which also confirmed the predominantly parasite-specific dysregulation of genes by the MetAP2 inhibitor AGM-1470 (Supplementary figure 2). Note that in these experiments the proportion of infected and uninfected host cells varied with time point and compound treatments, which complicated comparative analysis of host gene expression. We have therefore not performed any detailed analysis on host gene expression beyond determining the number of affected genes in the host transcriptome.

There was a high degree of identity between genes that were dysregulated by the various differentiation inhibitor treatments (Figure 5C, 5D). Supplementary Table 2 contains the fold changes of parasite genes that met the threshold of statistical cutoff (FDR <0.1). As the nine differentiation inhibitors act upon eight different target proteins, the uniquely dysregulated genes are highly likely to be compound specific effects. In contrast, as all our nine tested compounds causes the same effect of sexual differentiatiaon inhibition, commonly dysregulated genes by multiple treatments might

be more involved in regulating the sexual differentiation. In line with this reasoning, we compiled a list of parasite genes that were dysregulated with the majority (at least five or more) of the compound treatments. We compared this gene list with the list of significantly dysregulated genes identified through RNA-seq between *C. parvum* asexual and female stage parasites separated by flow cytometry. Our commonly upregulated gene set was highly similar to the gene set overexpressed in the asexual stage compared to female stage and our commonly downregulated gene set had a high degree of similarity with the genes overexpressed in the female parasites (Supplementary figure 3), which validates our dataset and our approach of selecting stage-specific genes.

2.3.5: Female-specific Genes are Down-regulated and Asexual Stage-specific Genes are Upregulated Following Treatment with Differentiation Inhibitors

Life cycle stage specific gene expression is a well-documented phenomenon in apicomplexan parasites. We wanted to check if the dysregulated genes from the compound treated samples were stage specific or not. To putatively identify stage specific genes, we have used two relevant RNA-seq datasets available in the EUpathdb database (31), one for *C. parvum* and the other one for *Plasmodium*. The *Plasmodium* dataset compared gene expression patterns between the asexual blood stage, and male and female gametocytes (18). We selected *Plasmodium* genes that are differentially expressed between these stages (FDR<0.1; log2 fold change of ≥1). Genes that are upregulated in the asexual stage compared to gametocytes were denoted as asexual

stage specific genes. Genes that are upregulated in both gametocytes or in one of the gametocytes compared to asexual stage were denoted as gametocyte, male and female specific genes accordingly. Ortholog transformation of each of these gene sets identified their homologs in the *C. parvum* genome, and was used to enable comparison of *C. parvum* and *Plasmodium* data. The Eupathdb *C. parvum* dataset contains the gene expression pattern for FAC sorted asexual stage parasites and female gamonts (7). Using the same cutoffs for differential expression, we identified asexual and female specific genes using this dataset. If any gene was putatively identified as asexual stage specific or male specific based on the *Plasmodium* database but marked as female specific from the *C. parvum* dataset or vice versa, we denoted them as divergent genes. All the other genes without any stage specific expression were denoted as unresolved. This set of genes most likely encode proteins that perform essential functions throughout the life cycle of *C. parvum*.

After using these existing datasets to classify all of the *C. parvum* genes as above, we checked if there was any stage specificity in the differentially expressed genes identified following compound treatments. All the compounds showed a similar pattern in our analysis; asexual stage specific genes were upregulated and female stage specific genes were downregulated due to compound treatment (Figure 5E, Supplementary Figure 2). We did not observe any strong effect in the other classes of genes.

2.3.6: Proteins Involved in Ribosome Formation are Upregulated in Response to Differentiation Inhibitors

We performed functional annotation enrichment analysis of the upregulated and downregulated gene sets following treatment with all of the differentiation inhibitors using DAVID Bioinformatics Resources 6.8 (32). As there was a high degree of similarity between gene sets dysregulated by the different compound treatments, the functional annotation enrichment analysis identified similar gene classes enriched with all the treatments (Supplementary Table 3). Therefore, to limit the analysis to common pathways involved in differentiation and exclude potentially misleading effects seen with individual compounds, we repeated the analysis with gene sets significantly dysregulated (FDR<0.1) following treatments with the majority of the compounds (five out of nine treatments). We noticed that for the genes that were significantly dysregulated with multiple treatments, the log2 fold-change was often marginally below 1 for some treatments, so this cutoff likely resulted in the erroneous exclusion of some biologically relevant differentially expressed genes in the combined gene sets. Therefore, we bypassed setting a fold change cutoff for selecting commonly dysregulated genes and selected them only based on the FDR cutoff, which provided a better representation of all the different compound treatments. Following treatment with differentiation inhibitors, this analysis indicated that genes encoding proteins associated with ribosome biogenesis and structure were commonly upregulated, whereas genes encoding proteins involved with biosynthesis of secondary metabolites

and glycolysis were commonly downregulated (Figure 6A).

We wanted to check whether the upregulation of ribosomal genes was just a response to compound treatment or represented biologically relevant differential expression between *C. parvum* life cycle stages. For this, we reanalyzed the Eupathdb *C. parvum* dataset comparing the female and asexual transcriptomes to determine whether the functional annotation enrichment analysis of our dataset correlated with that dataset or not (7). As compound treatment inhibited sexual differentiation, genes upregulated with compound treatment should be downregulated in female gamonts when compared with the asexual stage and vice versa. Similarly, genes involved in glycolysis should be upregulated in female gamonts. Analysis of the existing *C. parvum* dataset confirmed these predictions (Figure 6A). Furthermore, ribosomal genes were the most significantly downregulated gene class in female gametocytes, strongly suggesting that modulating ribosome formation could be a key regulatory mechanism for parasite differentiation.

We plotted the actual average transcript per million (TPM) values of all the genes that code for proteins that are structural components of the ribosome. This indicated that mRNAs encoding this class of proteins reached their peak concentration at 36 hpi and then decrease rapidly at 48 and 72 hpi (Figure 6B). Compound treatment inhibited the repression of most of the members in this gene set. Interestingly, hierarchical clustering of the expression pattern of this gene set clustered the compound treated samples (prepared from 48 hpi) with 18 hpi and 36 hpi control samples whereas

the 48 hpi control sample clustered with 72 hpi control sample (Figure 6B).

Ribosomal protein genes are scattered across the *C. parvum* genome, yet the mRNA levels were highly correlated with each other across time points and compound treatment. This prompted us to search for conserved sequence signatures within the upstream and downstream regions of these genes. Differential enrichment analysis of the upstream region of the ribosomal protein genes identified a DNA motif, BYGTCTC, that was highly enriched in these genes compared to all the annotated protein coding genes of *C. parvum* (Figure 6C). Other than the ribosome structural proteins (n=78), the motif was found in the promoter region of 455 additional genes. We checked if those genes were consistently upregulated or downregulated (dysregulated in >=5 compound treatment), and, based on a Chi-square test, the motif was more commonly found in the promoter region of genes upregulated following treatment with differentiation inhibitors (Figure 6D). Furthermore, analysis of the motif's location indicated that it is typically located closer to the start codon for genes coding for ribosomal structural proteins and genes that were consistently upregulated, and is generally distally placed for the genes that were downregulated following compound treatment (Figure 6E). A comparison of the downstream sequences of ribosomal genes with other *C. parvum* genes did not identify significantly enriched sequence motifs.

2.3.7: Ribosomal Proteins are Generally Downregulated in Response to Translational Repression Programs During Differentiation in Apicomplexan Parasites

Our mRNA-seq dataset indicated that ribosomal protein genes are downregulated during *C. parvum* sexual differentiation. We checked if this is a common mechanism of regulating differentiation in apicomplexan parasites by using a variety of datasets available via Eupathdb to identify genes that are upregulated or downregulated in different life cycle stages of other apicomplexan parasites (Supplementary Table 4) (11, 18, 33-36). In *Plasmodium*, ribosomal genes are downregulated during female gametocytogenesis as well as in salivary gland sporozoites compared to midgut oocysts. In *T. gondii*, ribosomal genes are downregulated in bradyzoites compared to tachyzoites (Figure 6F). Interestingly, in all the three above mentioned differentiation axes, mechanistic studies indicate that post transcriptional repression of gene expression plays a critical role in the differentiation process. During maturation of *P. berghei* female gametocytes, overexpressed mRNAs are protected at the cytoplasmic P bodies, which also repress their translation (37, 38). In *Plasmodium* salivary gland sporozoites, inhibitory phosphorylation of eukaryotic translation initiation factor alpha (eIF2α) represses the translation of mRNAs (39). The *T. gondii* eIF2α is also observed to be phosphorylated in bradyzoites (40, 41). Collectively, these publications indicate that the repression of ribosomal proteins correlates with a translational repression program in *Plasmodium* and *Toxoplasma*. In

Eimeria, ribosomal genes are upregulated in merozoites compared to both sporozoites (the earlier stage) and gametocytes (later stages) (Figure 6F). Overall, in all the three apicomplexan parasites where sexual and asexual stage gene expression has been compared with RNA-seq, ribosomal proteins are enriched in gene sets that are downregulated in sexual stages.

2.3.8: The DNA Recognition Motif of Four ApiAp2 Transcription Factors is Significantly Enriched in the Promoter Regions of Commonly Downregulated Genes

Genes that are commonly downregulated with five or more differentiation inhibitor treatments are potentially overexpressed during or after sexual differentiation. Specific regulatory DNA sequences might control their sexual stage specific expression pattern. To identify such potential regulatory elements, we screened the promoter regions of these genes for significant enrichment of DNA motifs. We found that the motif "CATGCAWH" is the most significantly enriched DNA motif in the promoter region of these genes (Figure 7B).

The family of apicomplexan AP2 transcription factors (TFs) has been extensively studied in *Plasmodium* parasites and several of them have been implicated in regulating parasite differentiation in the course of its life cycle (21, 22). There are 18 putative ApiAP2 TFs in the *C. parvum* genome based on sequence similarity to the AP2 DNA binding domain (42). Previously, using DNA binding microarray, the putative DNA recognition motifs of several *C. parvum* AP2 TFs were identified (42).

Interestingly, the "CATGCAWH" motif that is significantly enriched in the promoters of our downregulated gene set bears a high degree of similarity to the recognition site of four different ApiAP2 TFs (listed in Figure 7A). Two of these TFs (cgd8_3230 and cgd2_3490) themselves are also significantly dysregulated by five or more differentiation inhibitor treatments (Figure 7A), which suggests that they might play key regulatory roles in controlling the expression of sexual stage specific genes in *C. parvum*.

2.4: Discussion

In this study, we have tested the ReFRAME library, a diverse set of biologically active compounds, for activity against the sexual and asexual phases of the *C. parvum* life cycle. We identified compounds with differing modes of activity against the two life cycle stages and performed detailed transcriptomic analyses of a set of compounds that selectively affect the parasite sexual differentiation. We identified that proteins involved in ribosome formation are transcriptionally downregulated as parasites differentiate into sexual stage and treatment with differentiation inhibitors negatively affects this mode of regulation. In addition, we found that an experimentally validated binding site of several *C. parvum* ApiAP2 TFs is significantly enriched in the promoter region of genes that are downregulated with multiple differentiation inhibitor treatments, which indicates that these TFs play key regulatory roles in *C. parvum* sexual differentiation.

The compounds in the ReFRAME library were initially developed to treat a

variety of diseases, with a bias towards cancer therapeutics. Respiratory, cardiovascular, and CNS conditions are also highly featured amongst the disease indications; altogether these indications account for ~86% of ReFRAME compounds, which were primarily developed to treat human conditions not caused by infectious agents (26). Therefore, many ReFRAME compounds target human proteins or RNAs. As an intracellular parasite, *C. parvum* must be sensitive to the altered condition in the host cells. It is therefore highly likely that a lot of validated hits from our screen are acting on *C. parvum* by altering the host cell condition, without directly targeting parasite proteins. A prime example of such mode of action is the four identified compounds that likely inhibit parasite sexual differentiation by acting on mitochondrial function (Figure 3A). *C. parvum* does not contain a fully functional mitochondrion itself; instead, it harbors a relict mitochondrion without a genome that is termed a mitosome (43). Consistent with a primary effect on the host cell, the two of the mitochondrial inhibitors included in our RNA-seq analysis caused dysregulation of a high number of host genes in addition to parasite genes (Figure 5B). This strongly suggests that host cell mitochondrial function is critical for parasite life cycle progression and its dysregulation affects the parasite sexual differentiation more profoundly than general growth. Interestingly, media removal to the establish an air-liquid-interface (ALI) condition during the culture of stem cell derived mouse intestinal epithelial cells supports the continuous cultivation of *C. parvum* in vitro (44). Parasite vacuole number significantly increases under the ALI condition from two days onwards

post infection compared to non-ALI condition, and, in contrast to the HCT8 culture system, both fertilization and production of infectious oocysts occurs. Consistent with our finding that inhibitors of the host cell mitochondrian block *C. parvum* sexual development, host cell RNA-seq comparison of the ALI and non-ALI conditions identified oxidative phosphorylation as the most significantly upregulated pathway under ALI conditions (44).

Targets of five differentiation inhibitors are enzymes of interconnected folate metabolism and purine nucleotide biosynthetic pathways (Figure 3A, 3B). We persued two of these in our follow up experiments, pralatrexate, an inhibitor of mammalian dihydrofolate reductase (DHFR) (45), and mycophenolate mofetil, a prodrug that is converted into mycophenolic acid, which inhibits host inosine-5'-monophosphate dehydrogenase (IMPDH) (46). Recently, it has been shown that knocking out several of the *C. parvum* proteins involved in nucleotide salvage, including DHFR-TS (cgd4_4460) and IMPDH (cgd6_20), has no effect on the replication of *C. parvum* (tested for 48 hpi) (47). However, unlike WT parasites, these mutants are susceptible to pharmacological inhibition of relevant host nucleotide synthesis. Interestingly, both of these enzymes are significantly upregulated at 72 hpi compared to 48 hpi in vehicle treated *C. parvum* infection, which suggests an increased need for nucleotides during the parasite sexual stage (Supplementary Table 2). Phenotypes of nucleotide salvage pathway knockout mutants of *C. parvum* suggest that the parasite has redundant pathways to synthesize pyrimidine nucleotides but not purine nucleotides, which makes

them comparatively more dependent on the host for purines. As a likely cause of this duplicity, it has been proposed that host derived purine nucleotides are directly imported as ATP that fulfills the energy demand of the parasites in addition to serving as nucleic acid building blocks (47). As discussed later, our data suggest that in the sexual stage, either energy demand is more or access to the host nucleotide pool is reduced compared to asexual *C. parvum*. This might be the reason why the sexual stages of the parasite are disproportionally affected by inhibitors of host purine biosynthesis compared to asexual stages. Interestingly, both of the retested nucleotide biosynthesis inhibitors affected sexual differentiation only when added at 3hpi and 12hpi, but failed to completely do so when added later (Figure 4B). Perhaps the host ATP pool is sufficiently depleted for inhibition of sexual differentiation only with prolonged exposure of these compounds.

AGM 1470 and beloranib are fumagillin analogs that covalently bind to human methionine aminopeptidase 2 (MetAP2) (48). MetAP2 is a bifunctional protein and one of its functions is to remove the N-terminal methionine from nascent proteins. Fumagillin analogs covalently bind to a histidine residue in the active site of MetAP2 and block this function (48). The active site histidine and the surrounding residues are conserved in *C. parvum* MetAP2. The other function of MetAP2 is to bind and protect the eukaryotic translation initiation factor 2 alpha (eIF2α) from inhibitory phosphorylation (49). eIF2α plays a critical role in the rate-limiting step of translation initiation, and the phosphorylation of eIF2α inhibits this activity (50). Several kinases

phosphorylate eIF2α in response to diverse types of stresses (51). Covalent binding of the fumagillin analogs does not directly alter this second function of MetAP2; however, they can indirectly enhance this function as the covalent modification of the protein enhances its stability (52, 53). Treatment with these compounds significantly dysregulated a high number of parasite genes, but, compared to the other differentiation inhibitors, a very small number of host cell genes (Figure 5B). Therefore, while we cannot formally reject the possibility that these fumagillin analogs affect *C. parvum* through effects on host metAP2, it is highly likely that their effect on parasite sexual differentiation results from inhibiting the parasite enzyme.

Consistent with this possibility, regulation of eIF2α phosphorylation is implicated in controlling stage differentiation in both *Plasmodium* and *Toxoplasma*. *T. gondii* eIF2α phosphorylation is necessary for tachyzoite to bradyzoite differentiation (40, 41, 54-56). In *Plasmodium*, several proteins required for the liver stage are actually transcribed in salivary gland sporozoites, but their translation is repressed (19, 57, 58). IK2, a kinase of eIF2α, is critical for this translational silencing (39, 59). Even though MetAP2 has been implicated in the protection of eIF2α, its role has never been specifically investigated in apicomplexan eIF2α phosphorylation regulation of stage differentiation. As MetAP2 inhibitors inhibit the sexual differentiation of *C. parvum* and the transcriptional signature of this differentiation inhibition mimics translational silencing in apicomplexan parasites (Figure 6F), it is likely that these fumagillin analogs inhibit *C. parvum* sexual differentiation by inhibiting parasite eIF2α

phosphorylation (Figure 8). In this model, normal sexual differentiation requires phosphorylation of parasite eIF2α, which turns on a translational silencing program in the parasites. AGM 1470 and beloranib bind and stabilize parasite MetAP2, which leads to increased protection of eIF2α from inhibitory phosphorylation. As a result, the translational silencing program fails to activate and sexual differentiation is inhibited.

Among the compounds that induced sexual differentiation or development, common putative targets include DNA topoisomerase (four compounds), cyclin dependent kinases (cdks, three compounds), and Aurora kinases (two compounds). And among other validated hits, two cdk inhibitors are classified as pan-inhibitors and two others as asexual inhibitors. Similarly, five putative topoisomerase inhibitors and two aurora kinase inhibitors are classified as asexual stage specific inhibitors (Supplementary Table 1).

The purpose of the RNA-seq experiments reported here was to identify sexual and asexual stage specific genes and pathways, some of which might be involved in regulating *C. parvum* sexual differentiation. We selected compounds with diverse structures and putative targets for this study, all of which cause the same effect, i.e. inhibition of sexual differentiation (Figure 3A). For each of the compounds, we selected a dose that highly and preferentially inhibits differentiation (Figure 3B). We reasoned that with such a high effective dose for a particular phenotype, dysregulated genes and pathways common in the majority of the treatments would be parasite life cycle stage specific elements, as opposed to compound specific dysregulated elements.

Indeed, there was a high degree of commonality in dysregulated genes (Figure 5C and 5D, supplementary table 2). According to our experimental design, asexual stage specific genes should be upregulated and sexual stage specific genes should be downregulated following treatment with the differentiation inhibitors. We compared the consistency of our approach with the previously published complementary approach of conducting RNA-seq on FAC sorted parasites using a female gamont-specific promoter driven reporter to separate asexual and female sexual stage specific parasites (7). To include the male stage specific gene expression in our comparison, we interpolated stage specificity of *C. parvum* genes from a similar FAC sorting-based dataset of *Plasmodium* (18). A high degree of correlation between our dataset and the two other datasets validates our approach (Figure 5E). At the same time, it also indicates that the sexual stage specific gene expression pattern is reasonably conserved between *Plasmodium* and *Cryptosporidium.*

Functional annotation enrichment analysis of commonly dysregulated genes identified (dysregulated in ≥5 compound treatments) glycolysis as the most highly enriched pathway in the downregulated gene set and ribosome biogenesis in the upregulated gene sets (Figure 6A, Supplementary Table 3). The result of this analysis is consistent with the same analysis performed for dysregulated gene sets between asexual and female gamonts (Figure 6B). In the absence of a fully functional mitochondrion, glycolysis is considered to be the main energy generating pathway for *C. parvum*. As our data indicate that this pathway is more active following sexual

differentiation, we propose two mutually non-exclusive models to explain this stage specific energy metabolism in *C. parvum*. One is that the energy demand in sexually differentiated parasites is simply higher compared to the asexual stages. That can arise from the necessity of producing residual bodies, a type of energy storage structure composed of carbohydrates and other metabolites that are observed in both macrogamonts and oocysts (60, 61). Another possibility is that the sexual stages are less efficient in importing energy equivalents from the host, which is compensated by increased energy production by the parasite itself. As mentioned above, it has been proposed that *C. parvum* might harbor transporters that directly take up ATP from the host cell (47). Stage specific differences in the abundance of such a transporter might reduce energy uptake from the host cell following sexual differentiation. Overall, each model is consistent with our observations that inhibition of host oxidative phosphorylation and purine biosynthesis both affect sexual differentiation.

The upregulation of ribosome biogenesis and structural proteins following differentiation inhibitor treatments suggests an overall lower rate of protein synthesis in sexually differentiated parasites (Figure 6A and 6B). This persuaded us to look for similar transcriptional dysregulation patterns in other apicomplexans, as translational repression is a well-documented mechanism of regulating stage differentiation in apicomplexans. In all three differentiation axes where translational silencing has been validated with genetic experiments, the same ribosomal dysregulation signature has been observed (Figure 6F). Collectively, this strongly associates the downregulation of

ribosomal genes in RNA-seq datasets with the establishment of a translational silencing program. Without further experimental validation, however, it is unclear if the downregulation of ribosomal genes is causative for translational silencing or if the establishment of translational silencing somehow causes the transcriptional downregulation of ribosomal genes. Downregulation of ribosomal proteins is also observed in *Eimeria* gametocytogenesis; however, no mechanistic experiment to determine the regulation of this differentiation has been published.

One important point to note here is that all the apicomplexan parasites obviously possess stage specific proteins that are overexpressed under these general transcriptionally silenced and impaired-ribosome-biogenesis conditions. How the expression of those proteins is enhanced under such a condition is an important research question. Perhaps the answer is contained in the fact that ribosomes are not homogenous structures that keep translating all the mRNAs at a constant rate. Rather, they are heterogeneous structures that vary in building blocks between cell types and conditions, and they can selectively translate certain mRNAs over others (62). It would be interesting to experimentally test if such "special ribosomes" play a key regulatory role in apicomplexan stage differentiation.

Differential enrichment of mRNAs between conditions can result from their altered transcription or stability. In our RNA-seq follow up analysis, we focused on transcriptional regulation by looking at the putative promoter regions of *C. parvum* genes. We searched for enriched sequence motifs in the promoter region on various

subsets of *C. parvum* genes that are connected by similar dysregulation patterns and/or are from the same pathway or protein class. We identified very significant enrichment of a DNA motif in the upstream region of the ribosomal structure genes (figure 6C). This suggests that their transcription is controlled by the same transcriptional regulators and putatively explains why these genes are dysregulated as a group (Figure 6B). The other significant promoter motif signal that we observed came from the genes that are downregulated in ≥5 compounds treatments (Figure 7B). Interestingly, a part of this motif is identical to the experimentally validated core binding site of four different ApiAP2 transcription factors. TFs of this class are well defined as key regulators of apicomplexan stage differentiation. In both *Plasmodium* and *Toxoplasma*, the binding sites of multiple AP2 TFs are similar. They often function antagonistically to each other and the expression levels of the genes that they regulate are essentially determined by the expression levels of the TFs (63-65). Among the four *C. parvum* AP2 TFS that bind to the "TGCAT" core, cgd8_3230 is significantly upregulated in six treatments whereas cgd2_3490 is significantly downregulated with eight different treatments (Figure 7B). Combining all this information, we propose that the expression of a set of sexual stage specific genes is activated by cgd2_3490 and repressed by cgd8_3230.

Among all the validated hits from our screening, we have only used the differentiation inhibitors in this study to better understand the genetic regulation of sexual differentiation of *C. parvum*. Adding the other classes of hits, specifically the

sexual differentiation and development inducers, into the mRNA-seq analysis pipeline may further assist in recognizing the key regulatory factors in parasite sexual differentiation. Moreover, there are still numerous open questions about the parasite life cycle. The different classes of compounds identified in this study can be utilized in diverse ways to answer some of those questions. For example, in an animal model or in a continuous in vitro culture system, differentiation inhibitors can be used to check if sexual differentiation is an obligatory step following several rounds of asexual replication, i.e. whether the parasite would die or continue to grow asexually with a blockade in sexual differentiation. Such an experimental design would address the druggability potential of *C. parvum* sexual differentiation and may also lead to the identification of tool compounds to facilitate continuous cultivation of *C. parvum* in a simpler culture system than is currently available.

Before concluding, we would like to mention that as the ReFRAME library is biased towards human protein targets, applying a similar screening methodology with a less biased library might identify compounds that would more specifically target *C. parvum* proteins. Additionally, other phenotypic patterns may potentially be caused by compound treatment that we have not yet captured. For example, we have not yet identified a selective sexual development inhibitor or a sexual stage specific inhibitor that would inhibit both sexual differentiation and development. The utility of such tool compounds would be immense for the mechanistic understanding of *C. parvum* sexual differentiation.

2.5: Material and Methods

2.5.1: In vitro *C. parvum* Infection Model

Human ileocecal adenocarcinoma (HCT-8) cells (ATCC; catalog# CCL-244) were used as host cells for *C. parvum* infection. The cells were passaged in modified RPMI-1640 medium (Gibco, catalog# A10491–01) supplemented with 10% heat inactivated fetal bovine serum (Sigma) and antibiotic solution (120U/mL penicillin and 120 ug/ml streptomycin; Gibco 15140-122). Cell culture passage number 9 to 36 were used for parasite infection. *C. parvum* oocysts were routinely purchased from Bunchgrass Farm (Deary, ID) and used for up to 6 months. To infect HCT8 cells, oocysts were excysted in vitro by treating them first with 10mM HCL (37C, 10 minutes) and then 2mM sodium taurocholate (16C, 10 minutes; Sigma T4009). Host cells are grown to 90% confluency in appropriate plates for infection. Infection media was same as HCT8 culture media except that it was supplemented with 1% serum instead of 10% and antifungal compound Amphotericin B (0.625 ug/ml; Sigma A2942) was added in addition to the antibiotic solution. Infected cells are incubated at 37C with 5% CO2.

2.5.2: Compound Screening and Dose Response Validation

Host cells were grown in 384 well plates and infected with 5500 oocysts per well. Following infection, compounds were added at appropriate timepoints using Biotek Precision microplate pipetting system. Cultures were washed with wash solution (0.1% tween 20 in 1X PBS) 3 times before fixing with 4% paraformaldehyde

and permeabilizing with 0.25% triton X-100. 1% BSA was used for blocking before staining with Anti DMC1 antibody clone 1H10G7 (hybridoma supernatant). Plates were incubatd at 4c for at-least 24 hours with the primary antibody before washing with the wash solution three times, with five minutes incubation between each wash. Subsequently, the culture was stained with 4ug/ml Alexa Fluor 568 goat anti-mouse IgG (Invitrogen, A-11004) and 1.33 µg/mL FITC-labeled *Vicia villosa* lectin (Vector Laboratories, FL-1231) in 1% BSA for at-least 1 h at 37 °C. Finally, 0.09 mM Hoechst 33258 (AnaSpec, catalog# AS-83219) stain was added to stain the nucleus for 10 minutes. Then the wells were washed with wash solution five times, with five minutes incubation between each wash.

The culture plates were imaged with Nikon Eclipse Ti2000 epifluorescence microscope equipped with automated stage movement, using an objective with 20X magnification and 0.45 numerical aperture. 5 by 5 field of views were captured for each well. Images were analyzed with an automated Imagej macro (66) to count the number of nucleus, total parasite and $DMC1^{+}$ parasites.

2.5.3: mRNA-seq Sample Preparation and Sequencing

HCT8 cells were grown to 90% confluence on 24 well plates and infected with an appropriate number of excysted oocyst (2, 1, 0.5 and 0.4 million for 18, 36, 48 and 72 hpi samples respectively) in 1 mL of infection media. Compound or DMSO was added at appropriate time points. Before RNA isolation, wells were washed 3 times with the wash buffer. Total RNA was isolated using the Qiagen RNeasy mini kit (cat

#74104). Messenger RNA enrichment and sequencing libraries were prepared using NEXTFLEX Rapid Directional RNA-seq Library Prep kit bundle (cat # NOVA-5138-10) following the kit manufacturer's protocol. The next-generation sequencing (75 Base pair single end) was performed in the Vermont Integrative Genomics Resource Massively Parallel Sequencing Facility and was supported by the University of Vermont Cancer Center, Lake Champlain Cancer Research Organization, UVM College of Agriculture and Life Sciences, and the UVM Larner College of Medicine.

2.5.4: mRNA-seq Data Analysis

Sequences were demultiplexed and provided in ".fastq" format by Vermont Integrative Genomic Resource core facility. The sequences were uploaded to the public server of the Galaxy web platform (usegalaxy.org) and all the subsequent analyses were performed in that server (67). Sequences were quality trimmed using Trimmomatic (68) and then aligned to a combined genome sequence file of Human genome (Gencode release 31, GRCh38, (69)) and *C. parvum* genome (Cryptodb release 44, C. parvum IowaII, (70)) using HISAT2 (28). Alignment file was sliced into host part and parasite part using the Slice tool and processed separately in subsequent operations. We used HTseq (29) to determine the number of reads aligned to each gene. Deseq2 was used to analyze differential expression between appropriate samples (30). Deseq2 result files were downloaded, relevant values of the genes that met the differential expression cutoff were combined in a Microsoft excel file.

2.5.5: Pathway Analysis and Gene Expression Heatmap Generation

Functional annotation enrichment analyses with *C. parvum* genes were performed in DAVID Bioinformatics Resources 6.8 (32, 71). Appropriate gene ids were copied as gene list and CRYPTODB_ID was selected as Identifier type. All *C. parvum* genes were used as background and all the other options were used as default. For other apicomplexan parasites, "analyze result" was used from an appropriate apicomplexan parasite database after selecting appropriate genes using gene id search (31). TPM values of *C. parvum* ribosomal proteins were used to generate expression heatmap as well as clustering of samples using shinyheatmap (72). For clustering, the Euclidean distance metric and the "complete" linkage algorithm were used.

2.5.6: *C. parvum* genes' Upstream and Downstream Sequence Analysis

1 kb upstream and 1 kb downstream region of all the *C. parvum* genes were uploaded in the usegalaxy.org server. All the *C. parvum* coding sequences were then used as adapter sequences in the cutadapter tool to remove any coding part from the up and downstream sequences of the *C. parvum* genes. Such regulatory sequences from the selected genes were used as inputs and regulatory sequences from all the *C. parvum* genes were used as background in the "DREME" tool (73) hosted at "MEME-suite" server (74) to identify significantly enriched motifs. Position of specific motifs at the regulatory sequences of *C. parvum* genes were determined using the "FIMO" tool (75).

2.6: Figure legends

Figure 1: Stage specific assay development and library screening. A) Two replicate experiments showing anti-DMC1 antibody staining is sensitive and specific for quantitative screening purposes. HCT8 cell monolayers grown in 384-well culture plates were infected with *C. parvum*, and then stained for the female gamont-specific marker DMC1 at 72 hours post infection (hpi). The $DMC1^+$ parasite ratio was calculated for the 72 hpi timepoint, along with the z' score for each replicate. N=56 for each replicate, 28 each for wells stained with (red) or without (blue) inclusion of anti-DMC1 antibody. B) Representative images of HCT8 cells infected with *C. parvum* and stained with flourescent conjugated lectin, anti DMC1 antibody and nuclear stain. C) Combined assay strategy, showing compound addition and imaging timepoints as well as the four different readouts. Each readout is normalized to the readout value of vehicle treated wells from the same plate to calculate the "percent inhibition" value. D) Statistics of the primary screening for all the four readouts, showing average and standard deviation (SD) of the percent inhibition of vehicle-treated wells and inhibitor control wells from all the screening plates, as well as all the ReFRAME library compounds. Note that the expected mean percent inhibition by ReFRAME compounds with no activity (the vast majority) is 0. Hits were identified as compounds with a percent inhibition differing from the mean for all compounds by over two standard deviations. The cutoff values (library mean ± 2 * Standard deviation) for hit identification are given in the bottom two rows. E) Heatmap showing the result pattern

of the primary hits (311 compounds). Compound activity values are z normalized across each readout using the mean and SD of percent inhibitions for all the compounds of the ReFRAME library.

Figure 2: Categories of the validated hits and example dose response curves. A) Pie chart showing categorization of different hits. 2B to 2L show typical dose response curves for compounds representative of the different categories. Each point is from a single biological replicate and each curve shows one of the four readouts, as indicated in the figure. B) Pan-inhibitor, C) False hit, D) Asexual stage inhibitors without any activity in the sexual development readout, E) Asexual stage inhibitors with mid-range efficacy in the sexual development readout, F) Differentiation inhibitor, G) Differentiation inhibitor with narrow selectivity, H) Differentiation inducer, I) Development inducer, J) Inducer of both sexual readouts, K) Compound class undetermined. For 2H and 2J, activity values in the differentiation readout are excluded for the doses with above 70% asexual inhibition at 72hpi, because at these concentrations very few parasites were present. L) The dose response curve of the single compound that induced high DMC1 expression at 48 hours. $DMC1^{+}$ parasite ratio at 48 hpi (which is not one of the four standard readouts) is shown in green along with the four other readouts for this compound only.

Figure 3: Differentiation inhibitors share common putative targets and they selectively inhibit sexual differentiation across a range of concentrations. A)

Putative targets of all the categorized differentiation inhibitors. (*) denotes targets in nucleotide biosynthesis pathways and (**) denotes mitochondrial oxidative phosphorylation inhibitors. The first nine compounds were selected for follow-up experiments due to a large concentration window to enable inhibition of sexual differentiation with minimal effect on parasite asexual growth. B) Simplified nucleotide biosynthesis pathway of human cells. Enzyme names are colored and only enzymes with putative modulators in our validated hits are shown. Inhibitors of (*) marked enzymes are differentiation inhibitors and others are asexual stage inhibitors. Red colored enzymes have identified homologs in the *C. parvum* genome while the blue colored enzymes do not have an identified homolog in the parasite. PRPP: 5-phosphoribosyl pyrophosphate; PPAT: phosphoribosyl pyrophosphate amidotransferase; GARTFase: Glycinamide Ribonucleotide Formyltransferase; GAR: glycinamide ribonucleotide; IMP: Inosine monophosphate; XMP: Xanthosine monophosphate; DHF: Dihydrofolate; DHFR: Dihydrofolate reductase; THF: tetrahydrofolate; DHO: dihydroorotate; DHOD: dihydroorotate dehydrogenase; OMP: orotidine-5'-monophosphate. C) Dose response curves of the nine differentiation inhibitors selected for follow up. Compounds were added at 3 hpi and imaged at 72 hpi. Partially overlapping concentration ranges were tested for each of them in 2 to 3 biological replicates. Each point and error bar denotes mean and standard deviation for readings from 4 separate wells for each concentration in each biological replicate. The dotted reference line in the Y axis denotes the concentration selected for

mRNA-seq experiments.

Figure 4: Differentiation inhibitors are required to be added before parasite sexual differentiation. A) Experimental setup showing compound addition timepoints and measured response. B) Dose response curves showing percent inhibition of the proportion of $DMC1^{+}$ parasites at 72hpi after the addition of differentiation inhibitors at indicated timepoints. Each point and error bar denotes mean and standard error of the mean from 4 replicate well.

Figure 5: Treatment with differentiation inhibitors dysregulates a set of core genes involved in parasite differentiation. A) Experimental setup for the pilot mRNA-seq experiment. The number of genes dysregulated (fold change ≥2 at a False Discovery Rate of ≤ 0.1) between subsequent timepoints of the experiment are listed. B) Experimental setup and the number of dysregulated genes (fold change ≥2 at a False Discovery Rate of ≤ 0.1) with differentiation inhibitor treatments. C) Percentage of the upregulated *C. parvum* genes in each treatment that is shared by upregulated genes with other differentiation inhibitor treatments. D) Percentage of the downregulated *C. parvum* genes in each treatment that is shared by upregulated genes with other differentiation inhibitor treatments. E) Stage specificity of the genes that are dysregulated in ≥5 differentiation inhibitor treatments.

Transcriptome data was generated from 2 biological replicates for each

timepoint and/or treatment, except for 48 hpi control (n=3) and pralatrexate treatment (n=1).

Figure 6: *C. parvum* sexual differentiation is associated with suppression of ribosome formation, which is indicative of a translational repression program. A) DAVID functional annotation results for significantly dysregulated genes in ≥5 treatments, and between asexual and female *C. parvum*. Functional annotation enrichments with an FDR of ≤0.0001 are included only. Dysregulated genes were identified at a 10% false discovery rate (FDR) B) Heatmap of the average transcript per million (TPM) values of ribosomal protein genes of *C. parvum* at various timepoints (control) and in treatments with differentiation inhibitors (48 hpi). Each row represents a single gene encoding a ribosomal protein. TPM values are Z normalized across each row. Hierarchical clustering between the samples using the expression values of the ribosomal proteins was performed using the "complete" linkage algorithm and Euclidean distance metrics and is shown on the top of the heatmap. C) The most significantly differentially enriched DNA motif in the promoter region of the ribosomal protein genes compared to all *C. parvum* genes. D) Chi-square test of the association between the presence of the DNA motif from (C) in the promoter region of commonly dysregulated *C. parvum* genes (excluding ribosomal protein genes) and the direction of dysregulation. E) The location of the DNA motif from (C) in the promoter region of *C. parvum* genes harboring that motif. Other genes include genes that contain the motif in

their promoter region but are not dysregulated with ≥5 differentiation inhibitor treatment (fold change ≥1 and FDR ≤ 0.1) and not ribosomal proteins. F) Significant enrichment (p-value cutoff ≤ 0.5) of the two specific gene ontology (GO) terms during gene ontology enrichment analysis of apicomplexan genes that are dysregulated between the listed life cycle stages. Dysregulated genes are defined as genes with a log2 fold difference of ≥1 at 10% false discovery rate (FDR).

Figure 7: Promoters of downregulated genes of *C. parvum* harbor a recognition site for several ApiAP2 transcription factors that are also dysregulated by differentiation inhibitor treatments. A) log2 fold change of four ApiAP2 transcription factors of *C. parvum* with differentiation inhibitor treatments when FDR is ≤10%. B) The most significantly differentially enriched DNA motif in the promoter region of the genes that are ≥2 fold downregulated in ≥5 compound treatments compared to all *C. parvum* genes.

Figure 8: A proposed model of repressing *C. parvum* sexual differentiation by the two Methionine aminopeptidase 2 (MetAP2) inhibitors. Eukaryotic translation initiation factor 2α is abbreviated eIF2α. See the discussion section for detailed explanation of the model.

Supplementary Table 1: Structure, categorization, and putative mode of action

of all the compounds selected as hits from the primary screening.

Supplementary Table 2: log2 fold changes of parasite genes between different treatments and timepoints. Gene names from the Cryptodb database are included along with the gene IDs. Values are included only if the FDR is below 0.1 in differential gene expression analysis by DEseq2.

Supplementary Table 3: DAVID functional annotation enrichment analysis of dysregulated genes (fold change ≥2 and FDR ≤ 0.1) with differentiation inhibitor treatment.

Supplementary table 4: Eupathdb Gene ontology (GO) enrichment analysis of various life cycle stages of different apicomplexan parasites. In total, comparisons for 9 different axes of differentiation were performed; 5 from *Plasmodium*, 2 from *Eimeria*, and 1 from *Toxoplasma*. For each, both upregulated and downregulated genes are analyzed separately in "cellular component" and "molecular function", culminating in a total of 36 comparisons.

Supplementary Figure 1: Dose response curves of all the screening hits. 8-point dose response curves of all the primary screening hits in all 4 readouts. IDs correspond to the compound ids from the first column of Supplementary Table 1.

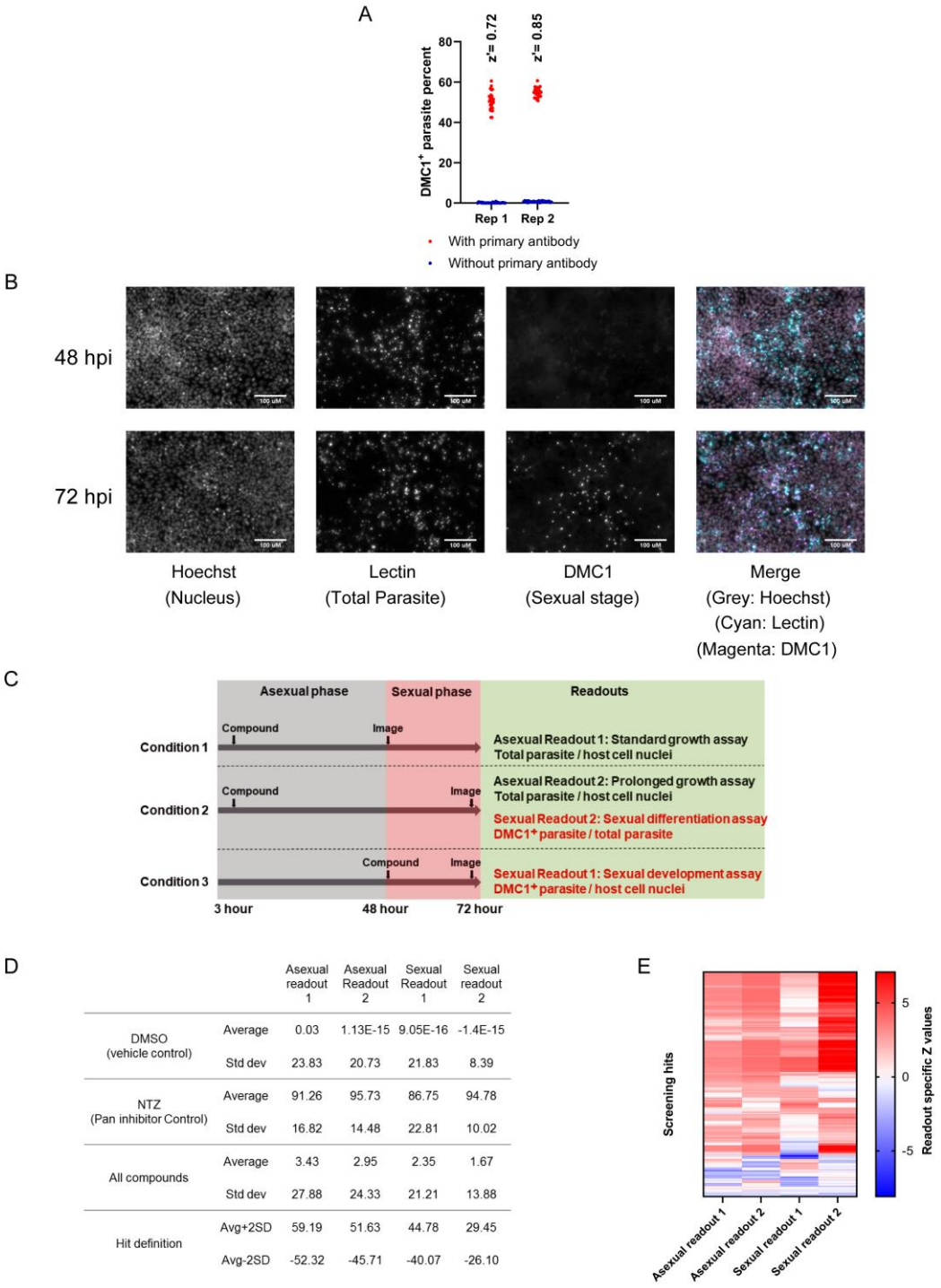
Duplicate curves (marked with "#") are included for compounds that induce sexual differentiation or development in a dose dependent manner at a concentration range that is not highly inhibitory to asexual replication. For the duplicate curves, relevant sexual stage readouts are excluded for high asexual stage inhibitory concentrations. IDs marked with asterisks (*) are compounds without a determined dose response in one of the readouts due to imaging quality error.

Supplementary Figure 2: Volcano plots of dysregulated genes by AGM-1470 and Oligomycin A treatment. *C. parvum* genes are shown with a circle and host genes are shown with a "plus" sign. Red colored genes are significantly differentially regulated and blue colored genes are not significantly differentially regulated. P-values were not calculated for the black colored genes.

Supplementary Figure 3: *C. parvum* asexual stage specific genes are upregulated and female specific genes are downregulated with differentiation inhibitor treatments. Two Venn diagrams showing the commonality between genes dysregulated with ≥5 differentiation inhibitor treatments with that of genes differentially regulated between FAC-sorted asexual and female stage parasites.

Supplementary Figure 4: Stage specificity of genes dysregulated by differentiation inhibitor treatments. The top panel shows the number of genes in

each class and the number of genes dysregulated by the treatment from that class. The bottom panel plots the log2 fold changes of significantly dysregulated (FDR $\leq$ 0.1) genes from the corresponding class.



		Asexual readout 1	Asexual Readout 2	Sexual Readout 1	Sexual readout 2
DMSO (vehicle control)	Average	0.03	1.13E-15	9.05E-16	-1.4E-15
	Std dev	23.83	20.73	21.83	8.39
NTZ (Pan inhibitor Control)	Average	91.26	95.73	86.75	94.78
	Std dev	16.82	14.48	22.81	10.02
All compounds	Average	3.43	2.95	2.35	1.67
	Std dev	27.88	24.33	21.21	13.88
Hit definition	Avg+2SD	59.19	51.63	44.78	29.45
	Avg-2SD	-52.32	-45.71	-40.07	-26.10

Figure 1: Stage specific assay development and library screening

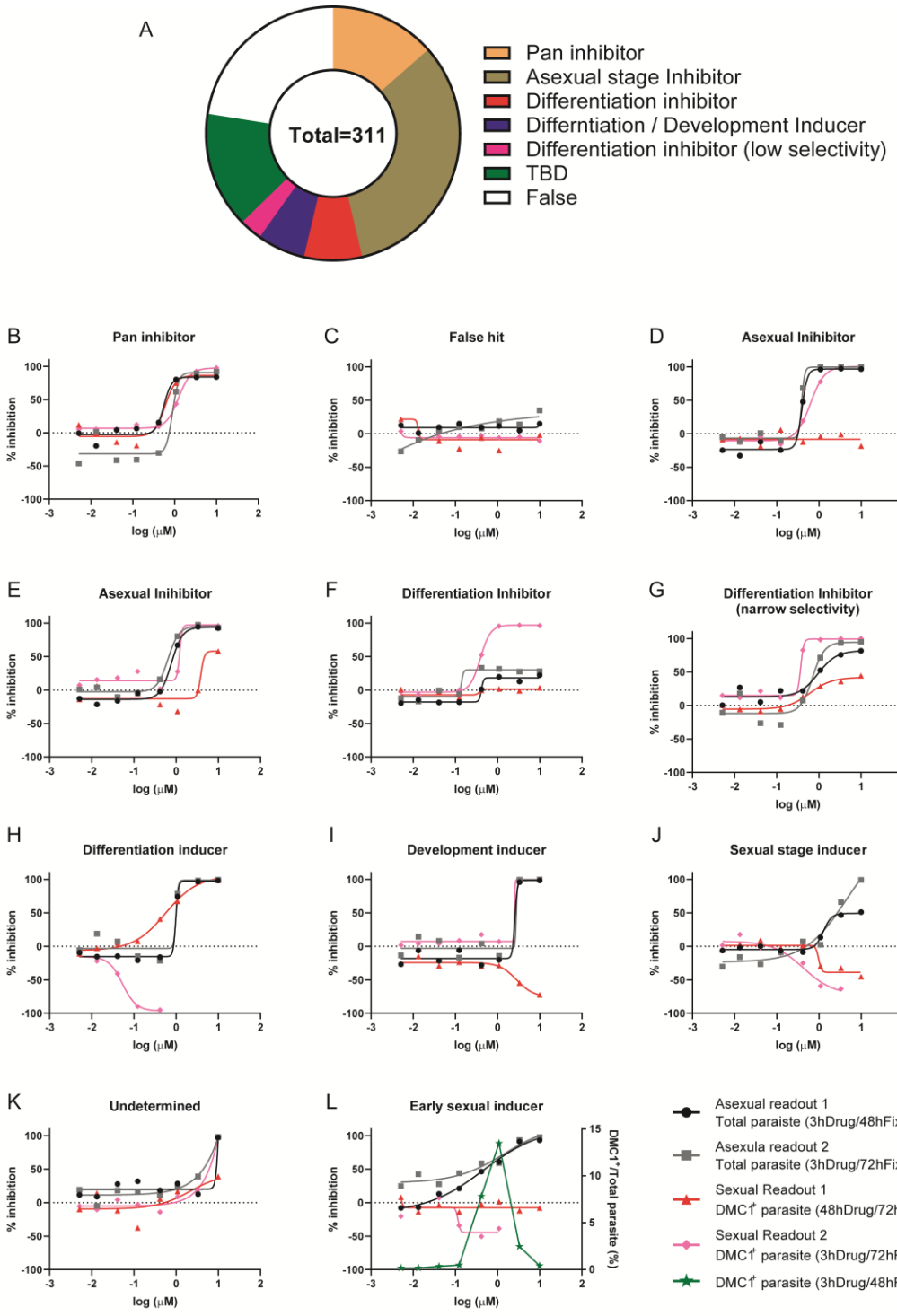


Figure 2: Categories of the validated hits and example dose response curves

A

Name	Putative target
Beloranib hemioxalate	Methionine Aminopeptidase-2 (MetAP2) Inhibitor
AGM-1470	Methionine Aminopeptidase-2 (MetAP2) Inhibitor
Oligomycin A	ATP synthase inhibitor**
Antimycin A	Mitochondrial complex III inhibitor**
BAY 61-3606 dihydrochloride	Tyrosine kinase inhibitor
Mycophenolate mofetil	Inosine 5'-Monophosphate Dehydrogenase (IMPDH) Inhibitors*
Pralatrexate	Dihydrofolate reductase (DHFR) inhibitor*
TVB-2640	Fatty acid synthase inhibitor
Mubritinib	ErbB-2 tyrosine kinase inhibitor
IACS-10759	Mitochondrial Complex I Inhibitors**
PYRIDABEN	Mitochondrial Complex I Inhibitors**
Metoprine	Dihydrofolate reductase (DHFR) inhibitor*
Methylthioinosine	Phosphoribosylpyrophosphatate amidotransferase (PPAT) inhibitor*
RWJ-46458	Angiotensin AT1 Antagonists
OCT-1547	RANK ligand inhibitor
Pelitrexol	Glycinamide Ribonucleotide Formyltransferase (GARTFase) Inhibitors*
BX-471	CC chemokine receptor 1 antagonist
CAI orotate	PI3 kinase inhibitor/ VEGF receptor antagonist/ Calcium channel antagonist
SCHEMBL10678365	Antidepressent
ZD 2138	Lipoxygenase 5 Inhibitor
FLUFYLLINE	5 Hydroxytryptamine 2 receptor antagonist

B

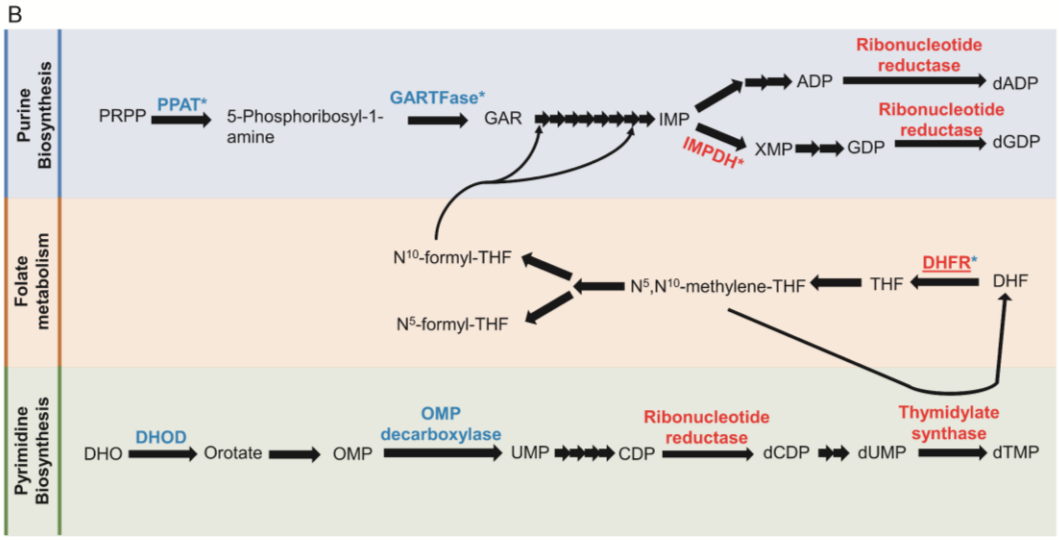


Figure 3: Differentiation inhibitors share common putative targets and they selectively inhibit sexual differentiation across a range of concentrations

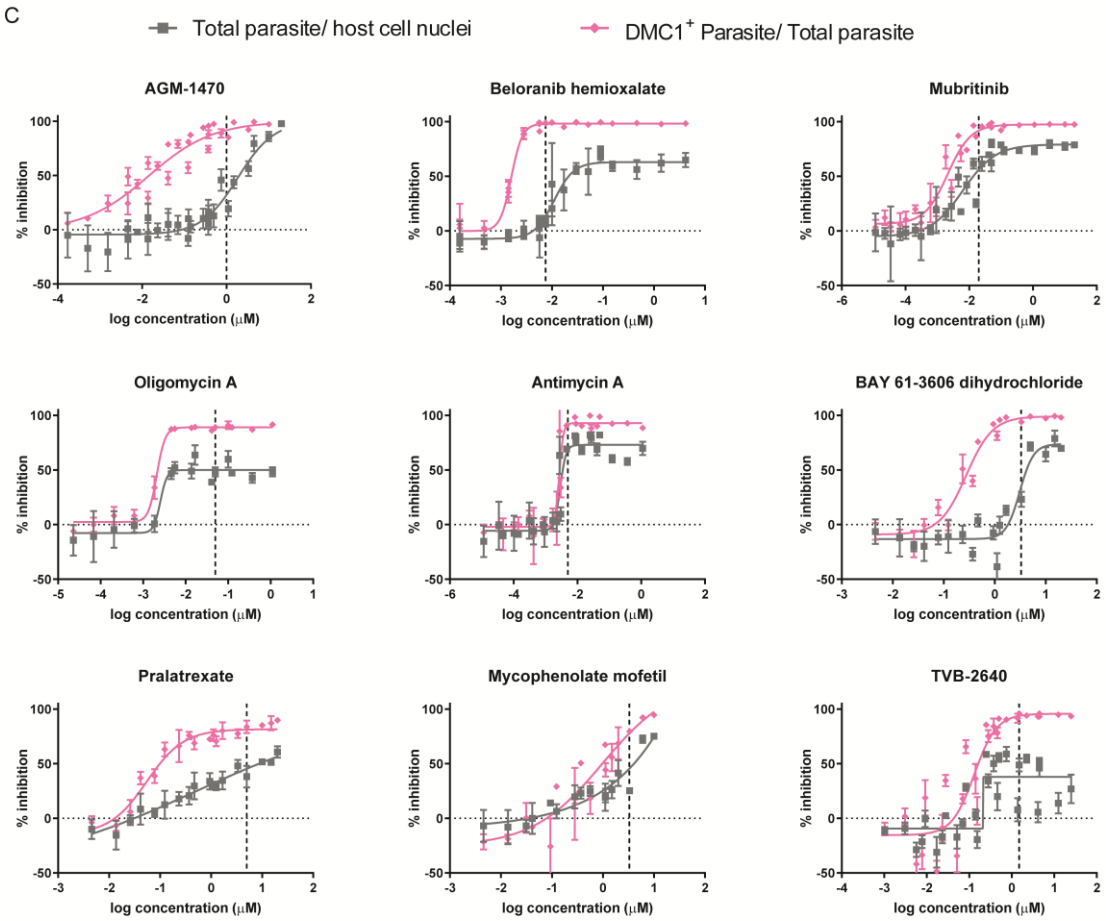


Figure 3 (continued)

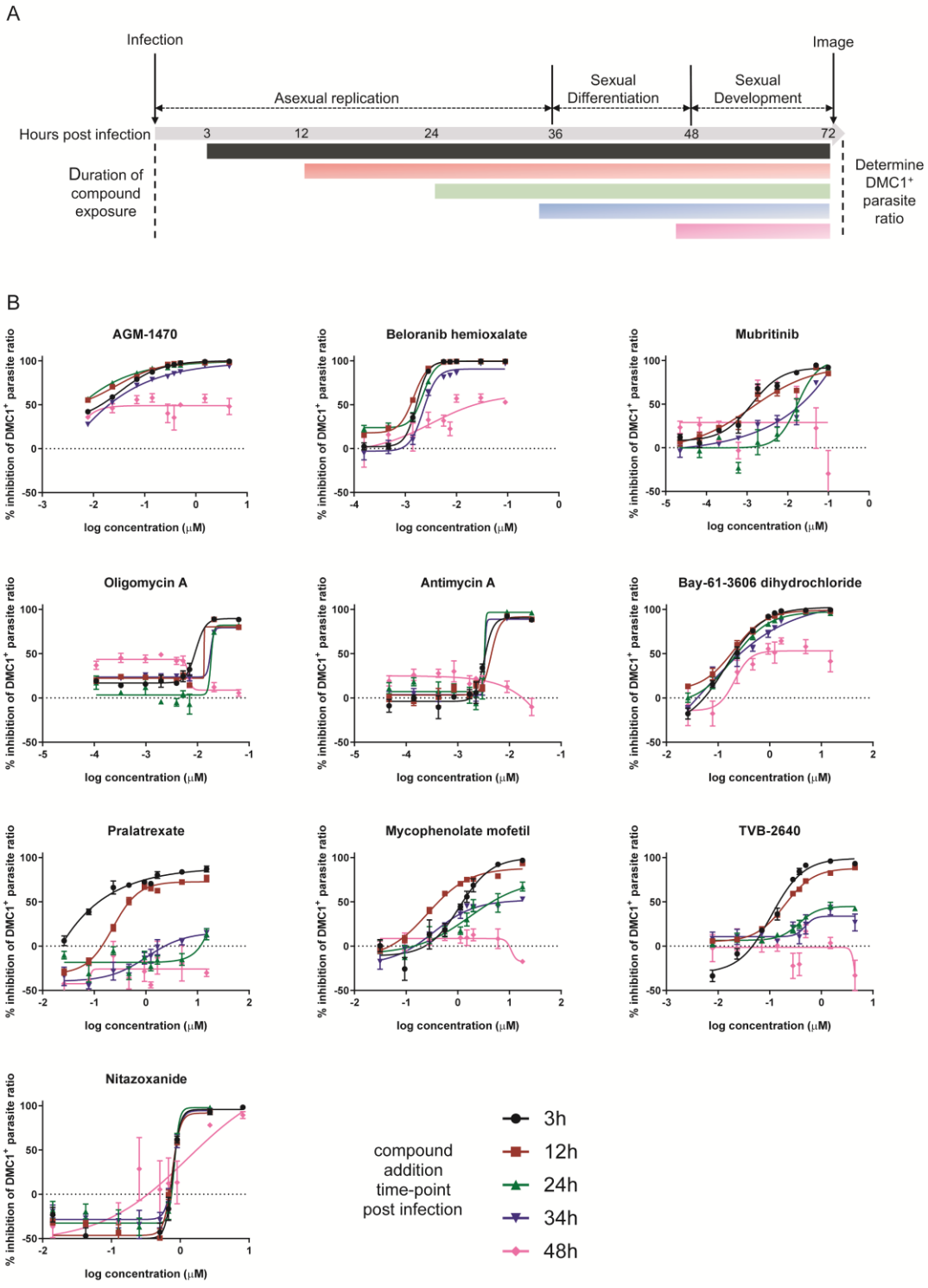
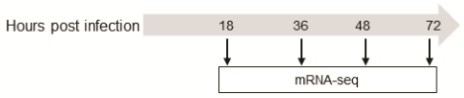


Figure 4: Differentiation inhibitors are required to be added before parasite sexual differentiation

A

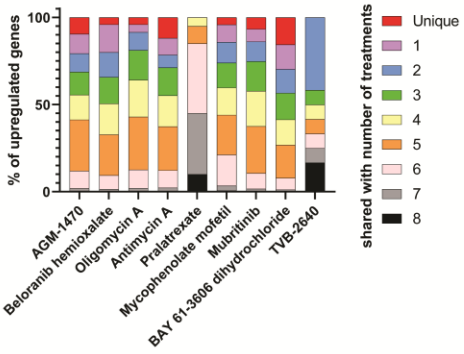


	18 to 36 hpi	36 to 48 hpi	48 to 72 hpi
Upregulated *C. parvum* genes	39	467	821
Downregulated *C. parvum* genes	1	38	635
Upregulated HCT8 genes	73	2	2
Downregulated HCT8 genes	3	1	25

B

	AGM-1470	Beloranib hemioxalate	Oligomycin A	Antimycin A	Pralatrexate	Mycophenolate mofetil	Mubritinib	BAY 61-3606 dihydrochloride	TVB-2640
Upregulated *C. parvum* genes	518	660	491	438	20	288	583	794	12
Downregulated *C. parvum* genes	591	768	721	872	197	661	739	1121	44
Upregulated HCT8 genes	0	1	2222	1459	74	647	1202	462	167
Downregulated HCT8 genes	0	2	3314	2276	14	362	2104	657	100

C



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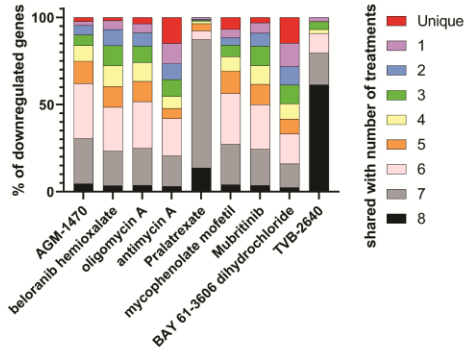


E

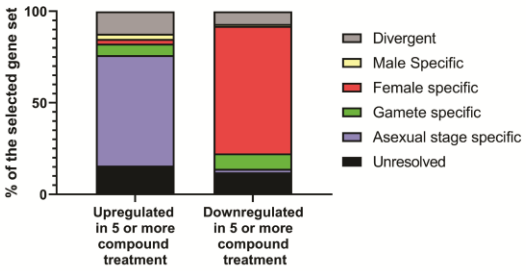


Figure 5: Treatment with differentiation inhibitors dysregulates a set of core genes involved in parasite differentiation

Comparison	Category	Term	P Value	Fold Enrichment	FDR
Upregulated in ≥5 compound treatment	UP_KEYWORDS	Ribosomal protein	8.0E-18	4.30	8.0E-17
	UP_KEYWORDS	Ribonucleoprotein	4.5E-16	3.74	4.4E-15
	KEGG_PATHWAY	cpv03008:Ribosome biogenesis in eukaryotes	1.7E-15	4.61	1.2E-14
	KEGG_PATHWAY	cpv03010:Ribosome	2.3E-15	3.50	1.6E-14
	GOTERM_CC_DIRECT	GO:0005840~ribosome	2.4E-15	4.36	2.0E-14
	GOTERM_BP_DIRECT	GO:0006412~translation	2.6E-14	3.90	2.6E-13
	GOTERM_MF_DIRECT	GO:0003735~structural constituent of ribosome	7.6E-14	3.99	7.9E-13
	GOTERM_BP_DIRECT	GO:0042254~ribosome biogenesis	3.5E-08	7.26	3.4E-07
	GOTERM_CC_DIRECT	GO:0005730~nucleolus	7.8E-08	6.54	6.6E-07
	GOTERM_BP_DIRECT	GO:0006364~rRNA processing	1.9E-07	5.94	1.9E-06
	UP_KEYWORDS	Ribosome biogenesis	9.3E-06	7.47	9.2E-05
Downregulated in ≥5 compound treatment	KEGG_PATHWAY	cpv01110:Biosynthesis of secondary metabolites	7.3E-08	3.64	7.2E-07
	KEGG_PATHWAY	cpv00010:Glycolysis / Gluconeogenesis	3.0E-07	6.30	3.0E-06
	KEGG_PATHWAY	cpv01130:Biosynthesis of antibiotics	1.7E-06	4.01	1.7E-05
	KEGG_PATHWAY	cpv01200:Carbon metabolism	5.0E-06	5.72	4.9E-05
Downregulated in female compared to asexual stages	UP_KEYWORDS	Ribosomal protein	9.9E-41	4.15	1.1E-39
	UP_KEYWORDS	Ribonucleoprotein	6.6E-39	3.77	7.1E-38
	GOTERM_CC_DIRECT	GO:0005840~ribosome	4.7E-32	3.75	4.6E-31
	GOTERM_MF_DIRECT	GO:0003735~structural constituent of ribosome	5.0E-32	3.68	5.9E-31
	GOTERM_BP_DIRECT	GO:0006412~translation	5.9E-32	3.41	6.6E-31
	KEGG_PATHWAY	cpv03010:Ribosome	2.0E-28	2.76	1.9E-27
	KEGG_PATHWAY	cpv03008:Ribosome biogenesis in eukaryotes	5.8E-07	2.15	5.4E-06
	GOTERM_BP_DIRECT	GO:0006351~transcription, DNA-templated	4.0E-06	2.36	4.4E-05
Upregulated in female compared to asexual stage	KEGG_PATHWAY	cpv01100:Metabolic pathways	3.5E-06	1.77	3.5E-05
	KEGG_PATHWAY	cpv00010:Glycolysis / Gluconeogenesis	8.6E-06	4.51	8.6E-05

B

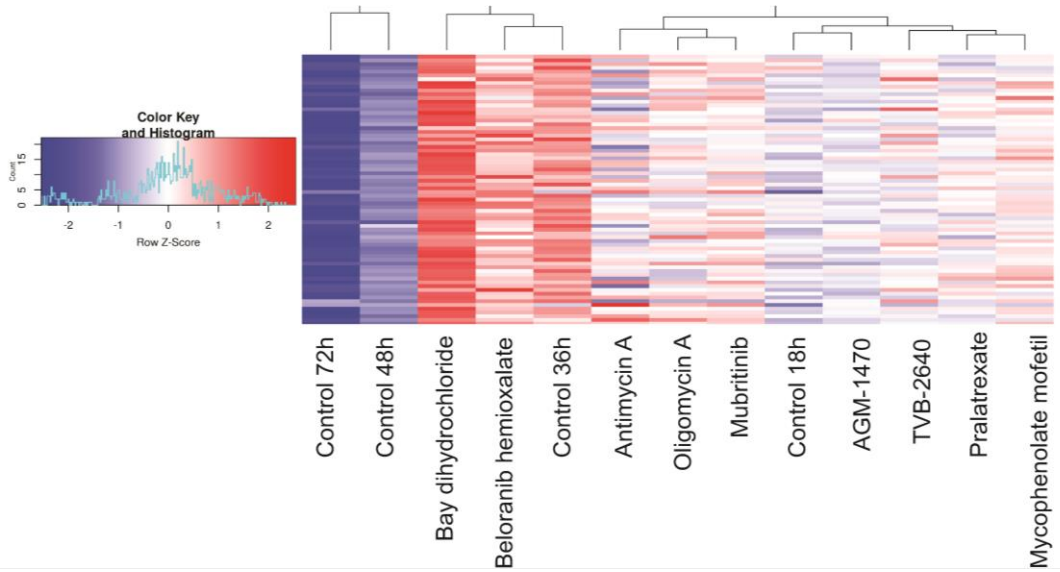
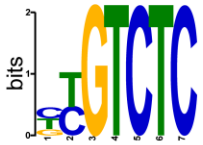


Figure 6: *C. parvum* sexual differentiation is associated with suppression of ribosome formation, which is indicative of a translational repression program

C



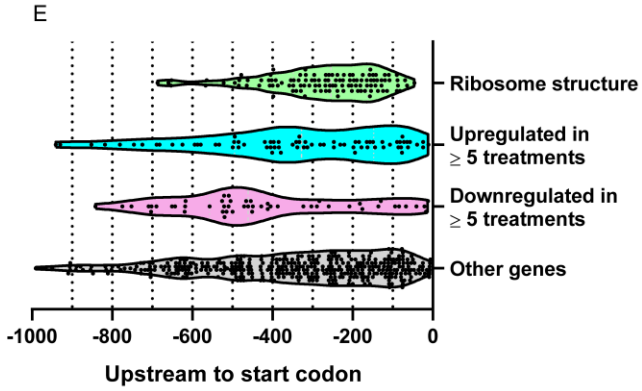
Present in
71 of 78 Ribosomal proteins
533 of 3989 *C. parvum* genes
Enrichment P-value: 3.9e-052

D

Contingency Table	"BYGTCTC" positive	"BYGTCTC" negative
Up in ≥5 treatments	69	277
Down in ≥5 treatments	44	529

Test	Chi-square
Chi-square, df	30.08, 1
z	5.485
P value	<0.0001
One- or two-sided	Two-sided

E



F

Organism	Comparison	Molecular function: Structural constituent of ribosome			Cellular component: Ribosome			Dataset reference
		Fold enrichment	P-value	Benjamini	Fold enrichment	P-value	Benjamini	
E. tenella	Downregulated in gametocytes compared to merozoites	2.44	2.31E-22	8.01E-20	2.46	6.75E-23	5.40E-21	11
E. tenella	Upregulated in merozoite comp to sporozoite	2.29	5.71E-21	2.60E-18	2.29	1.38E-20	1.32E-18	11
P. berghei	Downregulated in female compared to asexual stage	1.61	4.22E-05	1.95E-02	1.6	5.43E-05	2.78E-03	18
P. falciparum	Downregulated in sporozoites compared to oocysts	1.81	1.43E-10	2.96E-08	1.83	4.00E-11	1.07E-09	34
P. berghei	Downregulated in sexual stage compared to asexual stages	4.71	1.20E-05	1.56E-03	5.1	1.89E-06	1.76E-04	33
T. gondii	Downregulated in chronic brain infection compared to acute infection	6.08	4.48E-16	1.02E-13	6.03	5.58E-16	4.63E-14	35

Figure 6 (continued)

A

	cgd8_3230	cgd1_3520	cgd2_3490	cgd5_4250
Beloranib hemioxalate	0.94	0.82	-2.97	
AGM-1470	0.86	1.17	-2.20	
BAY 61-3606	1.15		-4.14	
Oligomycin A	1.20		-3.41	
Mubritinib	1.34		-2.88	
Antimycin A	1.95		-3.17	
Pralatrexate			-2.20	
Mycophenolate mofetil			-3.47	
TVB-2640				

B

Present in
291 of 573 downregulated genes (5 or more treatments)
1251 of 3989 *C. parvum* genes
Enrichment P-value: 2.2e-019

Figure 7: Promoters of downregulated genes of *C. parvum* harbor a recognition site for several ApiAP2 transcription factors that are also dysregulated by differentiation inhibitor treatments

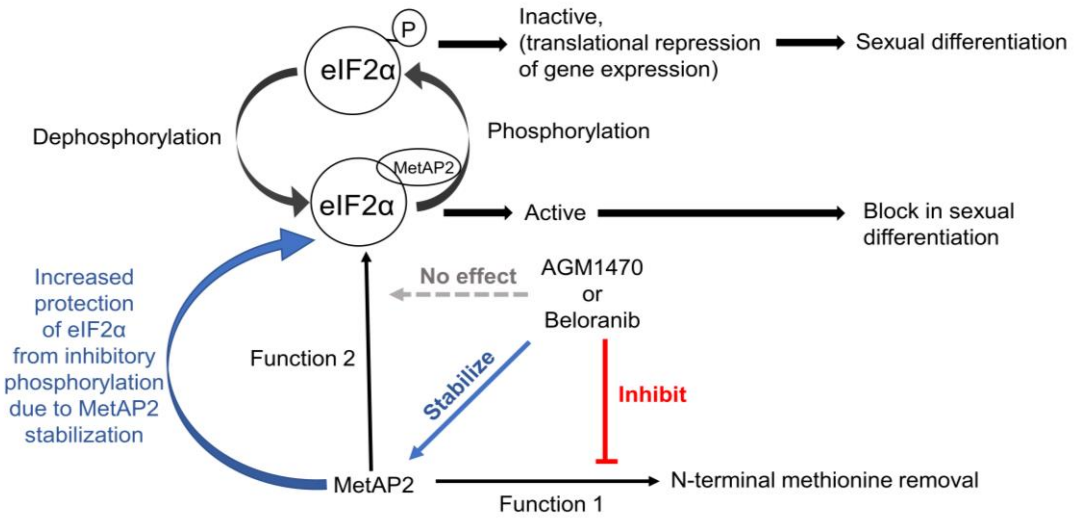
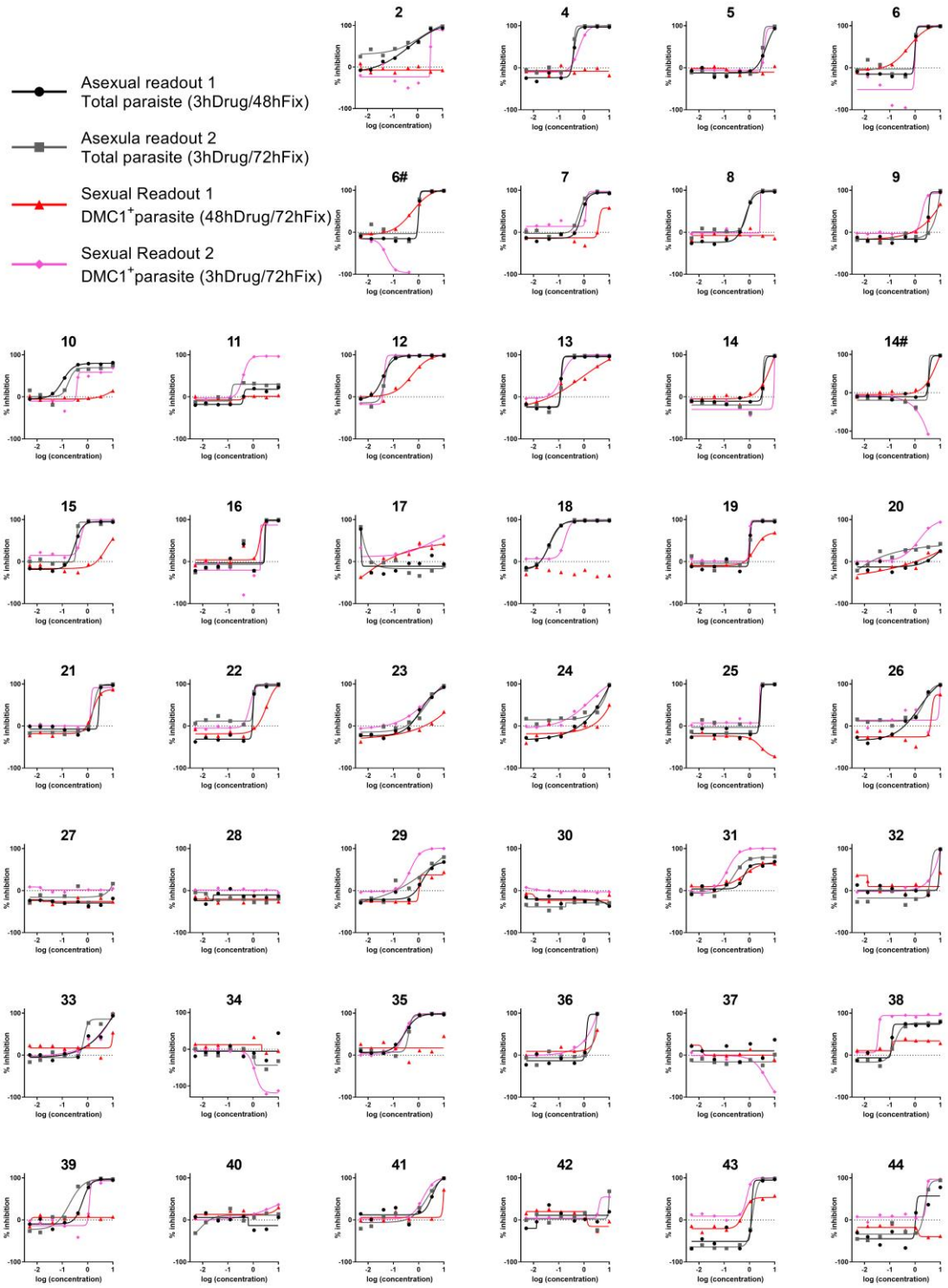
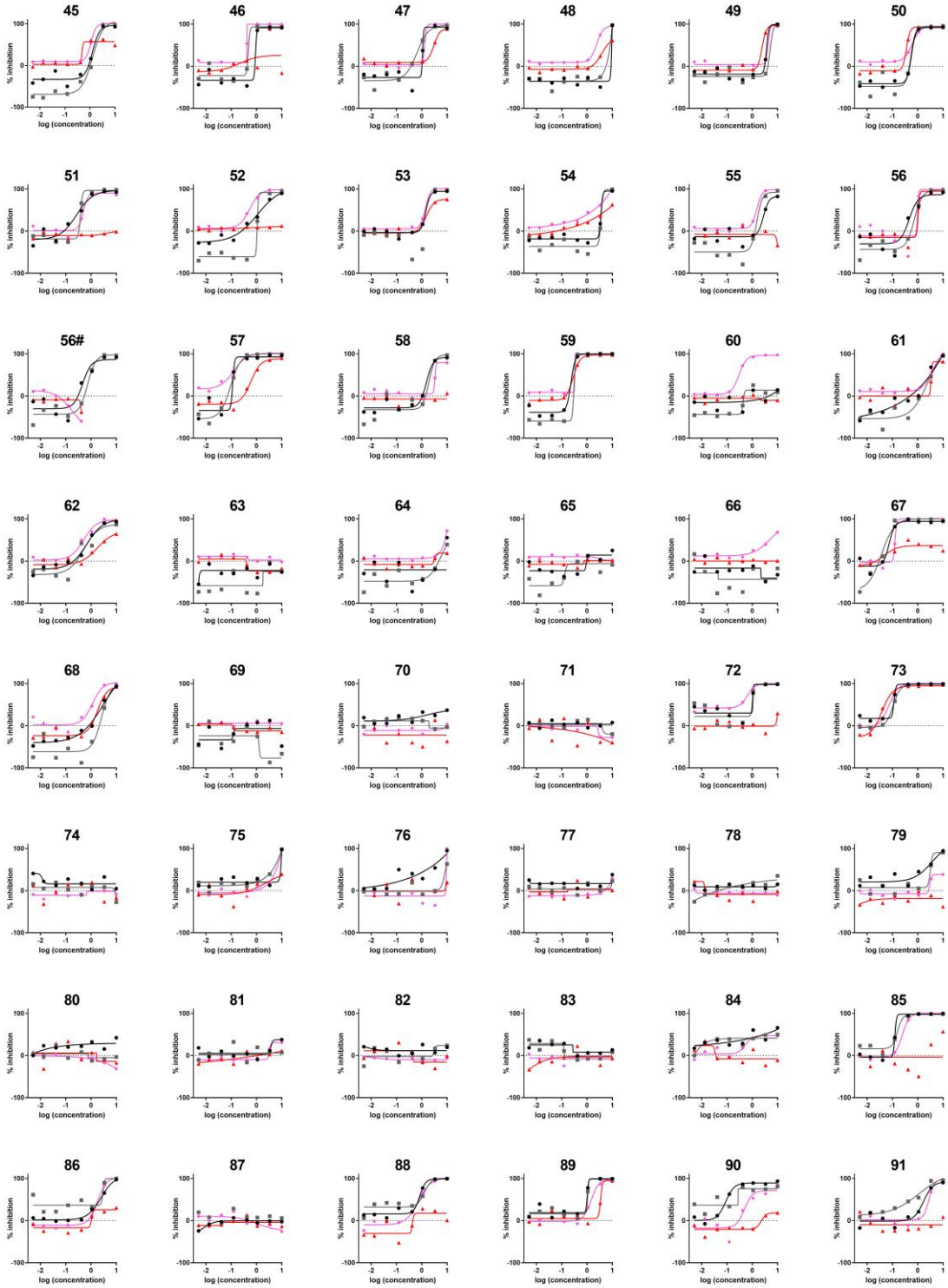


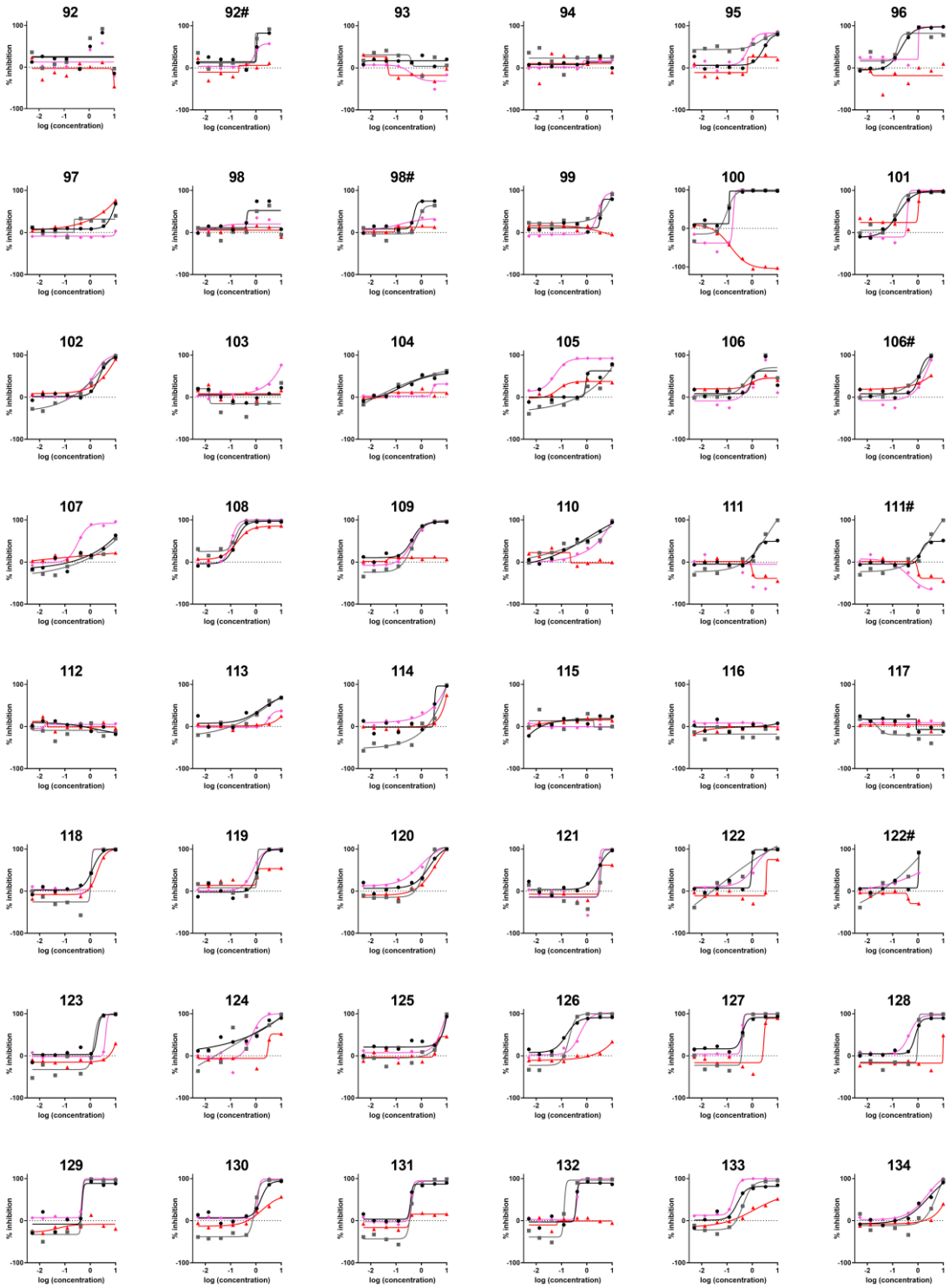
Figure 8: A proposed model of repressing *C. parvum* sexual differentiation by the two Methionine aminopeptidase 2 (MetAP2) inhibitors



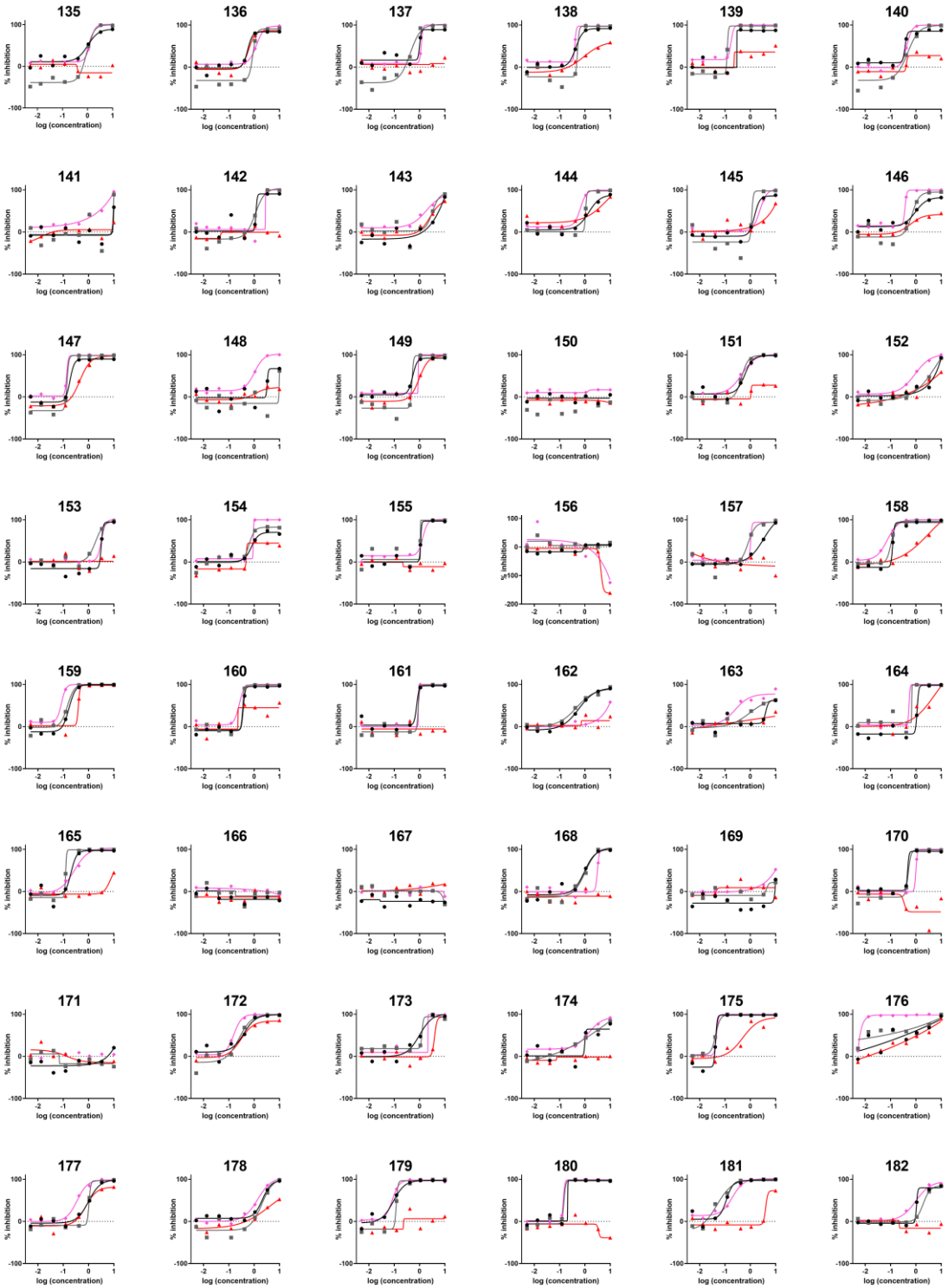
Supplementary Figure 1: Dose response curves of all the screening hits



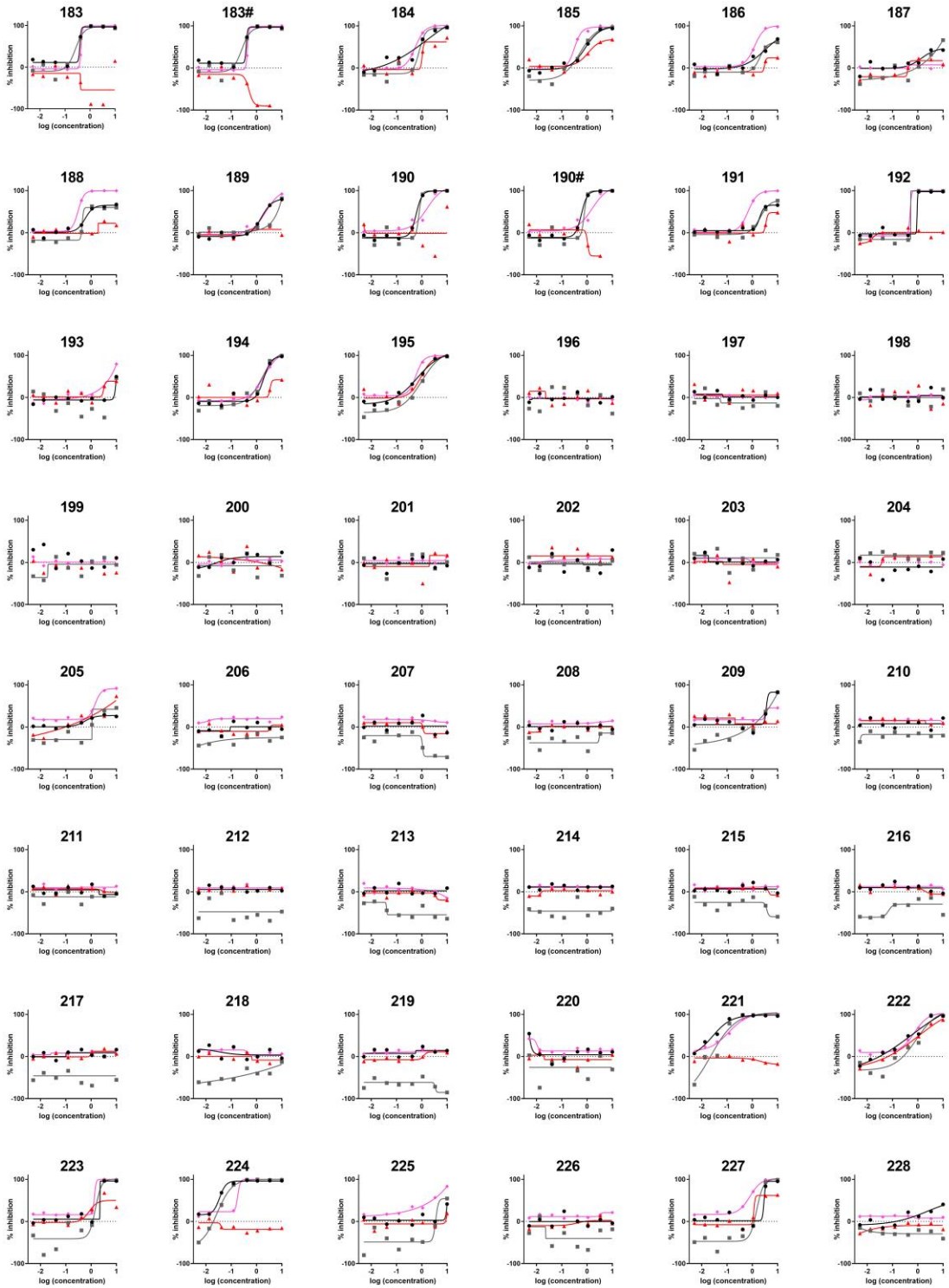
Supplementary Figure 1 (continued)



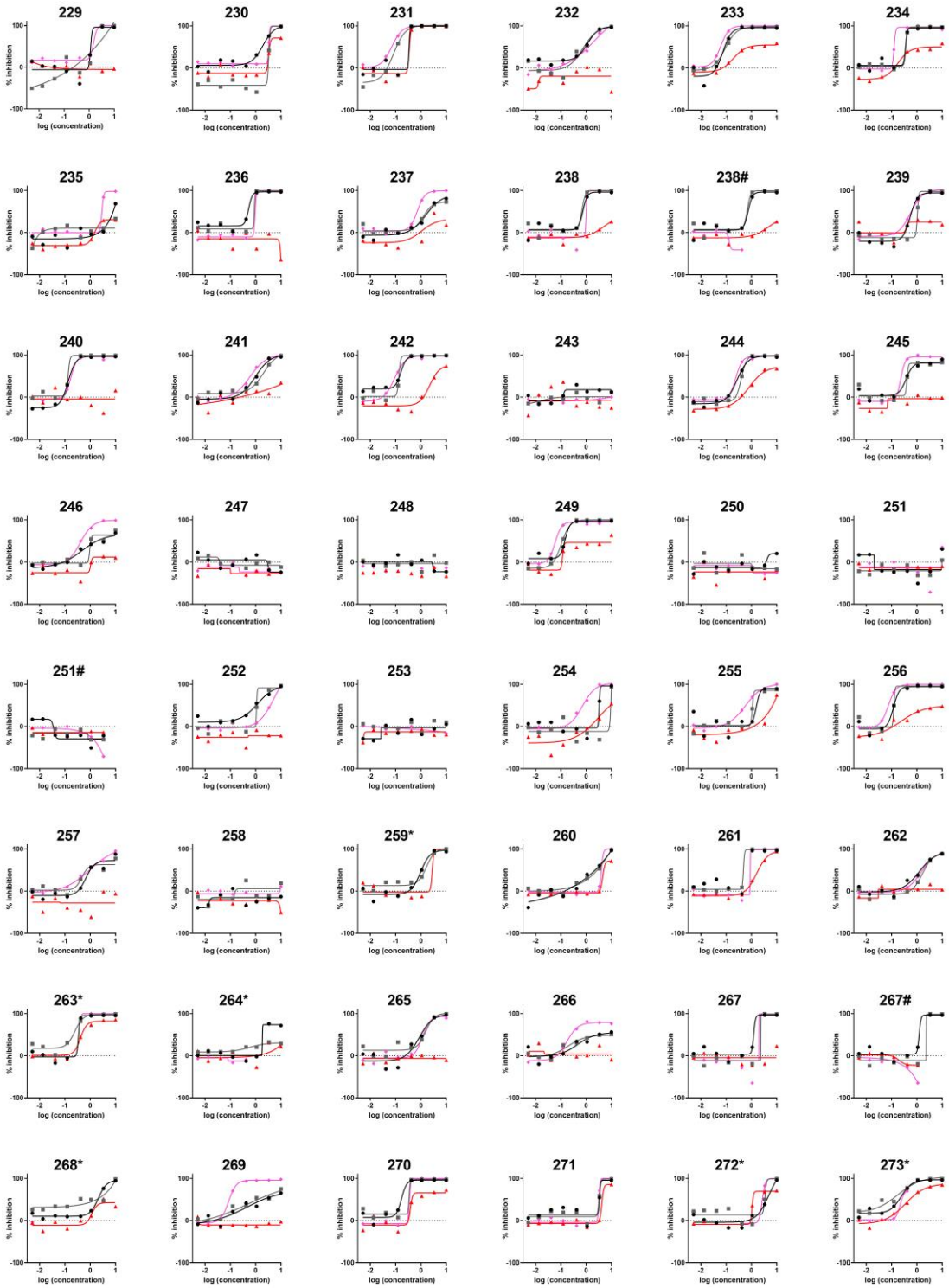
Supplementary Figure 1 (continued)



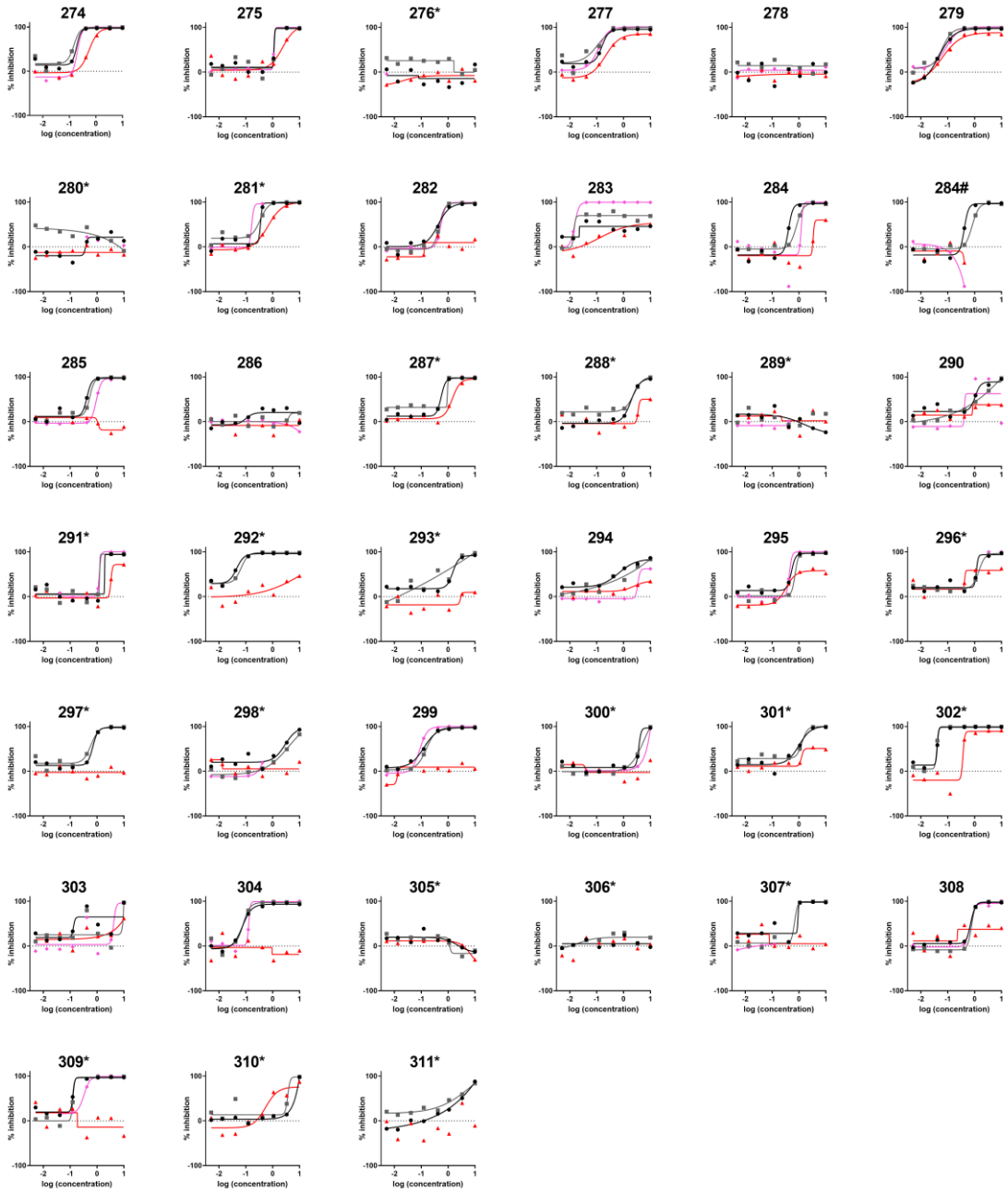
Supplementary Figure 1 (continued)



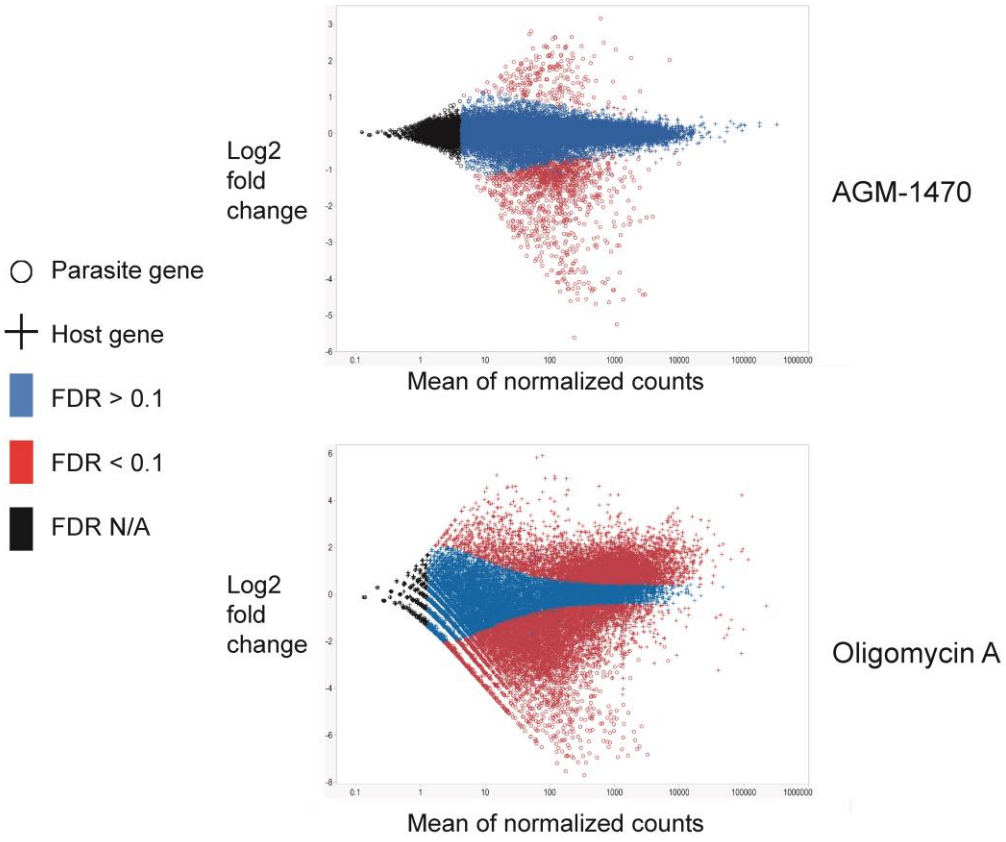
Supplementary Figure 1 (continued)



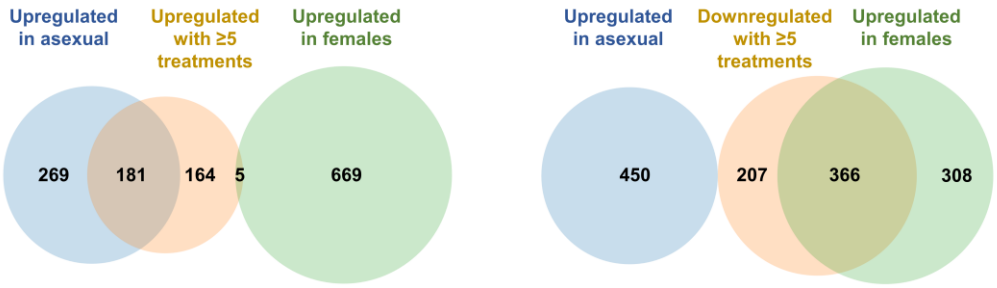
Supplementary Figure 1 (continued)



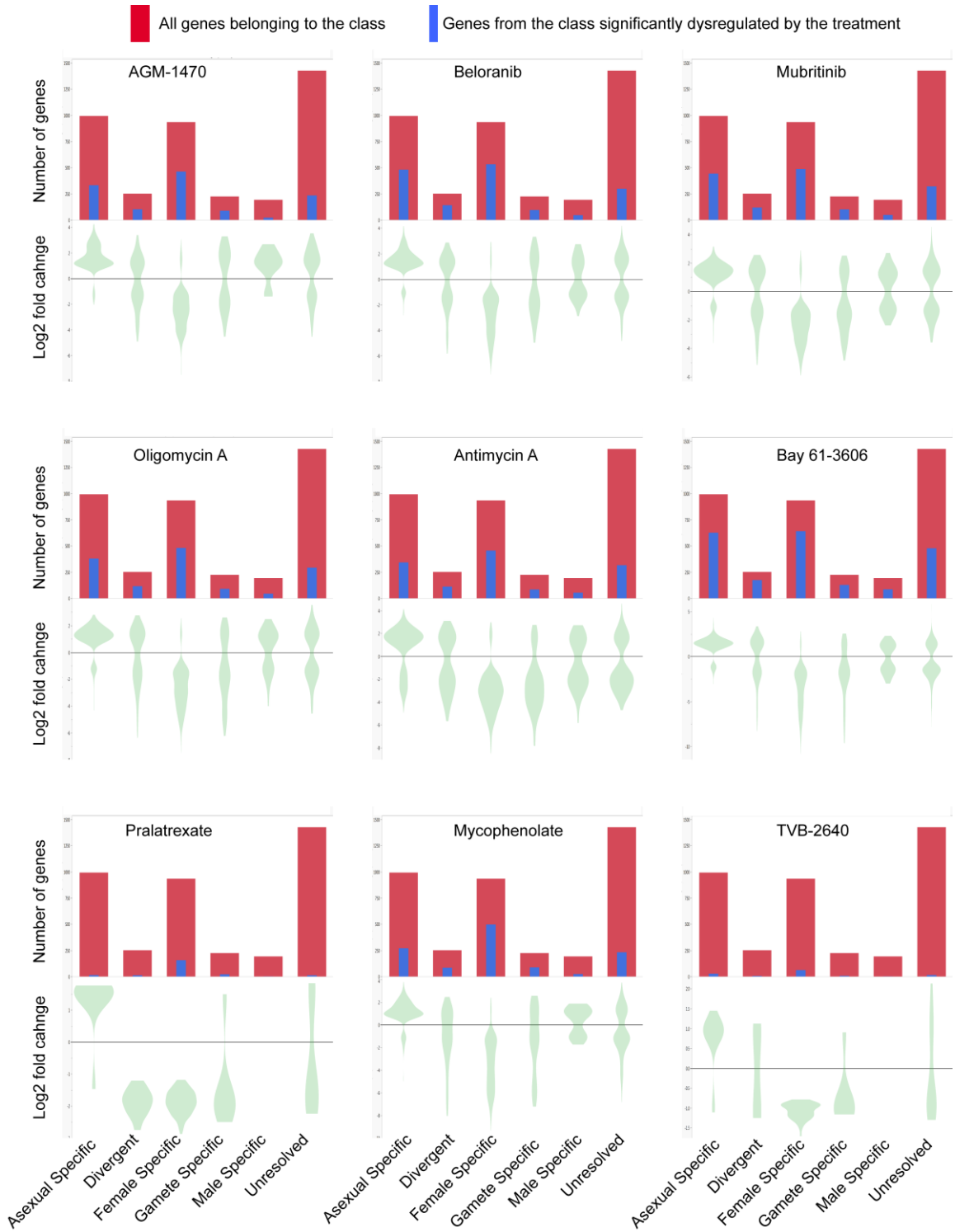
Supplementary Figure 1 (continued)



Supplementary Figure 2: Volcano plots of dysregulated genes by AGM-1470 and Oligomycin A treatment.



Supplementary Figure 3: *C. parvum* asexual stage specific genes are upregulated and female specific genes are downregulated with differentiation inhibitor treatments



Supplementary Figure 4: Stage specificity of genes dysregulated by differentiation inhibitor treatments

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CHAPTER 3: DISCUSSION AND FUTURE DIRECTION

Apicomplexan parasites, other than being huge public health concerns, are fascinating biological systems to study cell biology, parasitism, and evolution. These unicellular eukaryotic organisms are obligate intracellular parasites that are capable of morphing into distinct phenotypic structures throughout their life cycle. Epigenetic, transcriptional, and translational control of gene expression, undoubtedly shaped by the condition of the host cell or organism, are the key to achieving this extraordinary feat. In this study, we have used a chemical genomics approach to study the regulation of sexual differentiation of the apicomplexan parasite *Cryptosporidium parvum*. Our data suggest that (i) this differentiation is expectedly associated with large scale changes in the parasite transcriptome; (ii) modulating specific pathways of the host affects the differentiation process; and (iii) global translational control of protein expression likely mediated by altered ribosomal biogenesis plays a key regulatory role in controlling *C. parvum* sexual differentiation, which is perhaps a common mode of regulating stage differentiation in apicomplexan parasites.

3.1: Potential Function and Stage Specificity of the *C. parvum* DMC1 protein

We have used the expression of *C. parvum* DMC1 (Disrupted Meiotic cDNA, cgd7_1690) protein as a marker for the parasite sexual stage. Most eukaryotes have a homolog of DMC1 and it is a meiotic recombinase that facilitates homologous chromosome pairing during meiotic recombination (1). Rad51, which is also a

recombinase, is a paralog of DMC1 that is also present in most eukaryotes, including *Cryptosporidium*. A gene duplication event during early eukaryotic evolution gave rise to the DMC1 and RAD51 recombinase families (2). Knocking out DMC1 in *S. cerevisiae* arrests its growth at meiotic prophase (3) whereas homozygous DMC1 knockout mice are sterile (4). On the other hand, RAD51 knockout is embryonic lethal to mice (5). These and other studies suggest that in most eukaryotes, DMC1 preferentially functions during meiotic recombination whereas RAD51 takes part in both meiotic and mitotic recombination (1). Previously, using qRT-PCR, we showed that DMC1 expression peaks at 72 hours post infection (hpi) and correlates with the emergence of gametocytes in the culture, which was the basis of using DMC1 as a sexual stage marker (6). Our time-course mRNA-seq experiment also confirms this observation (Figure 1). Interestingly, RAD51 (cgd5_410) expression also peaks at 72 hpi; however, the level of upregulation from 48 hpi is patently more pronounced for DMC1.

Immunofluorescence microscopy using an anti-DMC1 monoclonal antibody identifies DMC1 in uninucleated parasites, strongly suggesting that it is specifically expressed in female gamonts (6). This conclusion is further supported by the observation that immunofluorescence staining of oocysts locates DMC1 within the oocysts, but outside of the sporozoites (Figure 2). That is because the female gamont containing parasitophorous vacuoles (PV) ultimately differentiate into oocysts following fertilization. We also attempted to perform immunoelectron microscopy of

C. parvum infected HCT8 using our anti-DMC1 antibody clone, but failed to localize the protein with the method. Perhaps the reason behind this is that the tertiary structure of the DMC1 protein is sensitive to alcohol treatment. During our initial staining optimization, we tested different fixatives for the staining and both ethanol and glutaraldehyde fixation resulted in the loss of anti-DMC1 staining.

Determining parasite stages via alternative methods suggests that the ratio of macrogamonts in the HCT8 infection model increases logarithmically between 36 and 48 hpi (7, 8), but then the ratio is relatively unchanged between 48 and 72 hpi (7). By contrast, the DMC1 mRNA level and DMC1+ parasite number increase dramatically between 48 and 72 hpi (Figure 1 and 2). Our interpretation of these observations is that the level of DMC1 protein within female gamonts increases to a detectable level as the gamont matures. Alternatively, it is possible that our specific culture condition delays the emergence of female gamonts or the other methods overcalculated female gamonts at 48 hpi. In that case, the low level of staining and the low mRNA level at 48 hpi compared to 72 hpi might arise from a lower number of female gamonts present at 48 hpi instead of the proposed low level of DMC1 expression by female gamonts. As we determined the number of DMC1+ parasites at 72 hpi and the ratio of DMC1+ parasites is consistent with the ratio of female gamonts at that timepoint determined by other methods, whichever of the above-mentioned explanations is correct does not carry too much significance regarding our subsequent conclusions. However, the implication of the first model, that there is a maturation phase of female gamonts that takes around 24

hours, is in itself an important potential aspect of *Cryptosporidium* biology that is worthy of further experimentation. Understanding the requirements and regulations for the female gamont maturation might give us clues regarding the basis of failed gamont fertilization in the HCT8 infection model.

Another protein that has recently been used as a macrogamont marker is *Cryptosporidium* oocyst wall protein-1 (COWP1, cgd6_2090) (7). Interestingly, its expression level is stably maintained after sexual differentiation and does not vary as dramatically as DMC1 between 48 hpi and 72 hpi (Figure 1). Perhaps the COWP1 is a marker of female gamont that is independent of its developmental stage, which further supports our first model of slow maturation of female gamonts.

3.2: The Rationale for Focusing on Differentiation Inhibitors for Follow-up Experiments

Dose response dependent validation of the screening hits from the ReFRAME library allowed us to classify compounds into several groups. A major initial goal of our screen was to determine if differentiation inhibitors could be used to achieve continuous asexual replication of *C. parvum* within HCT8 cells, which might allow us to establish a continuous asexual culture system of *Cryptosporidium*. Toward that goal, we assayed *C. parvum* growth after a prolonged incubation of 7 days post infection in the presence of the differentiation inhibitors. While the numbers of parasites observed at these late time points were higher for several treatments compared to control (Figure

3), we did not observe the maintenance of logarithmic growth that is generally observed up to 48 hpi. Our tentative conclusion from this set of experiments is that the differentiation inhibitors might induce additional rounds of asexual replication; however, the culture system ultimately becomes unfavorable for continuous parasite replication. Variation of this experimental setup, like testing other concentrations of the compounds or adding the compounds multiple times throughout the experiment might produce a different outcome. The reason we did not pursue this further was that we also could not successfully subculture parasites from an infected HCT8 monolayer to an uninfected monolayer, which in our view is an important prerequisite for the development of a continuous culture system. Instead, we moved forward with utilizing differentiation inhibitors as tools to examine the genetic regulation of *C. parvum* sexual differentiation.

3.3: Potential Follow-up Experiments with Other Compound Classes

The other classes of compounds were theoretically not compatible with our initial goal of establishing a continuous asexual culture. Therefore, we did not acquire representative compounds from other classes for follow up experiments. However, they would be valuable complementary compounds in terms of examining the genetic regulation of sexual differentiation. For example, treatment with differentiation inducers might dysregulate parasite genes in the opposite direction from what we have observed from the differentiation inhibitor treatments. We are currently in the process

of performing detailed dose response curves for some of the other compound classes. After that, we plan to perform mRNA-seq experiments with some of them, analyze the gene expression dysregulation pattern, and compare that to the dysregulation pattern of differentiation inhibitor treatments.

3.4: Other Potential Utilities of Differentiation Inhibitors

Recently, several in vitro culture platforms have been described that support the full *C. parvum* life cycle (9-13). Establishment of an air liquid interface (ALI) condition for mouse intestinal stem cell derived culture produces viable oocysts upon infection that can further excyst and cause additional rounds of infection (13). The ALI condition is associated with substantive genetic and metabolic alteration of host cells compared to non-ALI condition, which led to the suggestion that the host cells under ALI condition provide a better condition for *C. parvum* growth. Additionally, small molecules can be administered in a defined concentration in this culture system (8, 13). Collectively, this platform is superior to test several potential effects of differentiation inhibitors on the *C. parvum* life cycle compared to HCT8 infection model (where the life cycle is not completed) or in vivo animal infection (where it is challenging to maintain a specific concentration of compounds in the target tissue). Our initial attempts to establish a continuous asexual culture in the HCT8 cells might have failed because the host cell became inhospitable for supporting prolonged parasite growth. ALI culture can remove that variable and the effect of differentiation inhibitors would

be interpreted more clearly. Also, the druggability of the parasite differentiation process can be robustly tested with differentiation inhibitors on the ALI culture. If any of our identified differentiation inhibitors block sexual differentiation in ALI cultures in a similar way, inhibition of continuous parasite growth would mean that targeting sexual differentiation would be a viable means for treating cryptosporidiosis. We are currently in the process of establishing this culture system in our laboratory.

3.5: Potential Genetic Validation of The Chemical Genomics Approach

CRISPR/Cas9 dependent genetic modification of *C. parvum* was published in 2015, making the parasite genetically tractable (14). Currently, there is a single validated selectable marker gene. Therefore, genetic modification of multiple loci is not possible at this point. Currently available plasmids are not episomally maintained within the parasite, so transgene expression is only possible by integrating them into the genome. Thymidine kinase has been proven to be a non-essential gene in the parasite, and this locus has been used to integrate transgenes (13-15). Codon optimized nanoluciferase has been used to detect the transgenic parasites. Also, several fluorescent proteins have been successfully expressed and detected in *C. parvum*.

Genetic knockout and allelic exchange using the CRISPR/Cas9 based strategy has been successfully demonstrated (14, 16). Importantly, the generation of transgenic clones requires oocyst formation from transfected sporozoites. Therefore, if a protein is essential in any of the life cycle stages, there is a high chance that direct knockout of

the protein or mutations that affect essential functions of the protein would not be possible. Conditional knockout strategies have yet to be demonstrated for the parasite; however, a properly working Cre-loxP system has been demonstrated using two parasite lines, one constitutively expressing Cre recombinase and the another harboring a floxed site that inhibits expression of a fluorescent gene. Mating of the two lines allowed expression of the fluorescent protein, demonstrating a functional Cre-loxP system (7). This system would only be useful for conditional knockout of genes that are essential between zygote formation to oocyst formation, and can only be studied for a single generation. Inducible promoters or tunable protein translation strategies have yet to be established. Conditional knockdown of protein expression strategies would likely be appropriate for studying the functions of essential genes. Several such strategies have been successful in the related *Plasmodium* and *Toxoplasma* parasites, and these approaches have the potential to work with *C. parvum* also. For example, the integration of a riboswitch at the UTR of a target gene allows inducible knockdown of essential *Plasmodium* genes (17). Induction of the riboswitch by the exogenous administration of the ligand glucosamine results in target mRNA degradation.

Several implications from our screening and RNA-seq experiments can be tested by genetic experiments. Some of them are outlined below:

3.5.1: eIF2α Phosphorylation Plays a Key Regulatory Role in *C. parvum* Sexual Differentiation

Based on the putative mode of action of the methionine aminopeptidase 2 inhibitors, we proposed that phosphorylation of eukaryotic translation initiation factor alpha (eIF2α) induces parasite sexual differentiation (Chapter 2, Fig 8). Cgd7_5270 is the annotated eIF2α of *C. parvum* and sequence alignment with eIF2α from other organisms suggests serine 57 is the conserved phosphorylation site. Allelic exchange of the gene to make S57A and S57D mutants, followed by their phenotypic characterization, specifically their sexual differentiation efficiency, can potentially be tested with the existing genetic tools. If making one or both constructs become challenging (due to being potentially lethal mutation), the WT and mutated genes can be expressed as a second copy by integrating them in a nonessential locus of the parasite genome. If possible, the inducible expression of such constructs should be attempted. The expression pattern of proteins involved in ribosome biogenesis or structure in eIF2α mutant parasites could potentially elucidate the proposed link between translational repression and downregulation of ribosome biogenesis.

Knocking out *C. parvum* MetAP2 (cgd2_2480) followed by testing the sexual differentiation phenotype would be a good strategy to validate the mode of action of the MetAP2 inhibitors. The N-terminal lysine-rich domain of the mammalian MetAP2 has been implicated in the protection of eIF2α from inhibitory phosphorylation (18). A similar poly-lysine stretch is present in cgd2_2480. Therefore, substituting the native

locus with an N-terminal truncated version of MetAP2, which might be a more tolerant genetic modification, could also be used to address the same research question.

3.5.2: ApiAP2 Transcription Factors Regulate *C. parvum* Sexual Differentiation

The expression of several ApiAP2 transcription factors (TFs) was dysregulated with multiple differentiation inhibitor treatments. cgd2_2670 is the only ApiAP2 TF that is highly downregulated with all of the differentiation inhibitor treatments (Chapter 2, Supplementary Table 3). Among others, the putative binding sites of cgd2_3490 and cgd8_3230 are significantly enriched in the promoter regions of the downregulated genes. Each of these three TFs would potentially be interesting genetic knockout or inducible knockdown targets. Also, tagging these proteins with fluorescent proteins or epitope tags could be useful to validate their expression pattern through the parasite lifecycle. Alternatively fluorescent proteins could be expressed under the promoters of these genes. Among the various utilities of epitope tagging, CHIP-seq using anti-tag antibodies could be used to determine the regulome of these TFs.

3.5.3: *C. parum* DMC1 is a Female Specific Protein Involved in Meiotic Cell Division of the Zygotes

Analyzing the expression pattern of DMC1 with high temporal resolution would help in determining the development kinetics of macrogamonts. This could be done more comprehensively by live fluorescent imaging compared to staining fixed cells at different time points. Toward that goal, florescent tagging of the DMC1 protein or expressing fluorescent proteins under the DMC1 promoter could be attempted. If

successful, such parasite clones would also be useful in tracking sexual differentiation in the ALI culture system or animal model infections and serve as a complementary parasite clone to the already described COWP1-tdTomato parasite clone generated for a similar purpose (7). As the DMC1 protein supposedly functions post-fertilization during sporulation, the Cre-loxP based conditional knockout strategy could be applied for conditional knockout of DMC1. Complete or conditional knockout of the protein would help in its functional characterization. Specifically, it would be interesting to check whether RAD51 can cover for the loss of the DMC1 function.

3.6: Concluding Remarks

The dogmatic view about the *Cryptosporidium* life cycle is that sexual differentiation is an obligatory process. Classically, this view has been established from electron microscopy-based observations of in vivo infection as well as in vitro infection models where gametocytes emerge after a certain period following infection (19-21). Recently, life cycle stage typing using alternative methods also showed that in the HCT8 infection model gametocytes are the predominant parasite population at the later stage of the infection (6-8). In the ALI culture system, it has been reported that oocysts are produced in waves that are separated by time intervals when parasites potentially reset the infection cycle (13). A similar fixed number of asexual replication cycles has been also proposed for *Eimeria*, which most commonly undergo three rounds of asexual replication before sexual differentiation (22). This obligate sexual

differentiation model is fundamentally different from the two other well studied Apicomplexa, *Plasmodium* and *Toxoplasma* (23). In *Plasmodium*, a subset of the asexually replicating parasite population undergoes sexual differentiation, whereas *Toxoplasma* sexual differentiation only takes place within the definitive host, cats. Potential environmental cues for sexual differentiation have also been characterized for *P. falciparum* and *T. gondii* (24, 25). The implication of the defined number of asexual replication cycles in *Cryptosporidium* and *Eimeria* is that a genetic program can sense the number of asexual replications that the parasite has undergone at any given moment; however, molecular charecterization of such a sensory mechanism remains elusive. Alternatively, it is possible that some kind of environmental cue actually induces the differentiation process in *Cryptosporidium* and *Eimeria,* and that environmental cue reaches a threshold around the time the parasites complete the defined number of replication cycles. Conceptually, such an environmental cue could be a parasite generated quorum sensing molecule or depletion/fortification of a host factor. An in-depth mechanistic understanding of the regulation of *C. parvum* sexual differentiation would shed light on this interesting unanswered question about *C. parvum* biology.

In this study, we deduced from our RNA-seq analysis that ribosome formation is downregulated during *C. parvum* sexual differentiation and coupled this insight with the putative mode of action of the MetAP2 inhibitors. Another interesting insight that is supported both by our RNA-seq data and the putative identities of the targets of

several differentiation inhibitors is that the sexual stage of the parasite is a more energy-demanding stage compared to the asexual stages. The RNA-seq data supports this theory as it suggests that glycolysis is upregulated following sexual differentiation, which is the most critical energy producing pathway for the parasite in the absence of a fully functional mitochondrion. It is proposed from other studies that *Cryptosporidium* uptake energy equivalents from the host cells directly in the form of purine nucleotides. Therefore, our theory of increased energy demand in the sexual stage is also supported by the observation that inhibitiors of host cell oxidative phosphorylation and purine nucleoide biosynthesis, two pathways involved in producing the energy molecules that *Cryptosporidium* can steal from the host, also disproportionaltely affect the sexual stages of the parasite more. Targeted genetic experiments are needed to test the validity of this hypothesis.

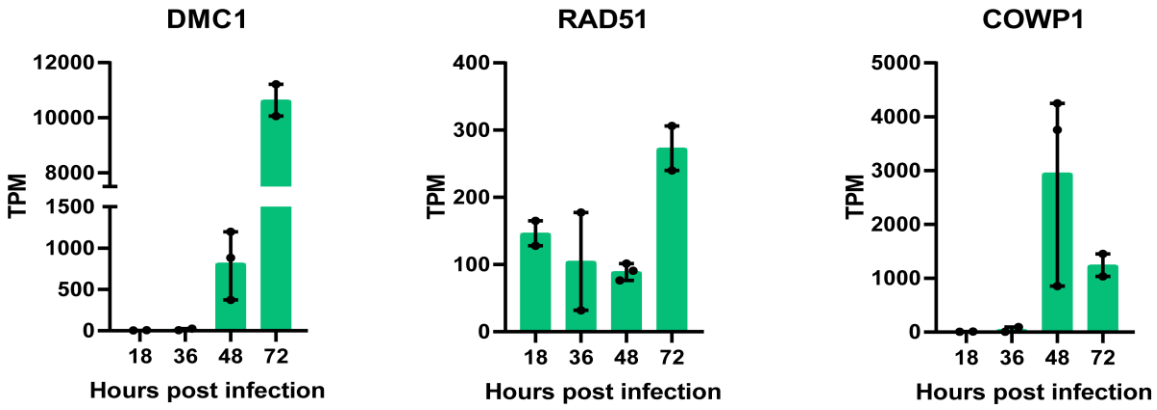

Figure 1: Expression time course of selected genes.

Transcript per million (TPM) values of DMC1, RAD51 and COWP1 mRNAs at different time-points post infection. Each point represents a biological replicate of mRNA-seq. Error bars denote range.

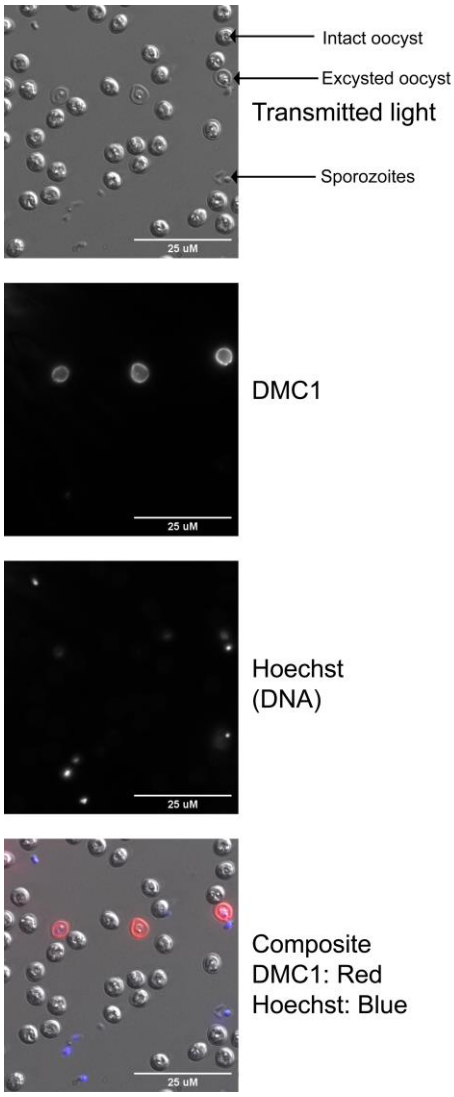

Figure 2: *C. parvum* oocysts stained with anti DMC1 antibody.

DMC1 protein is localized to the inner wall of *C. parvum* oocysts and is stained only when the oocysts are exysted.

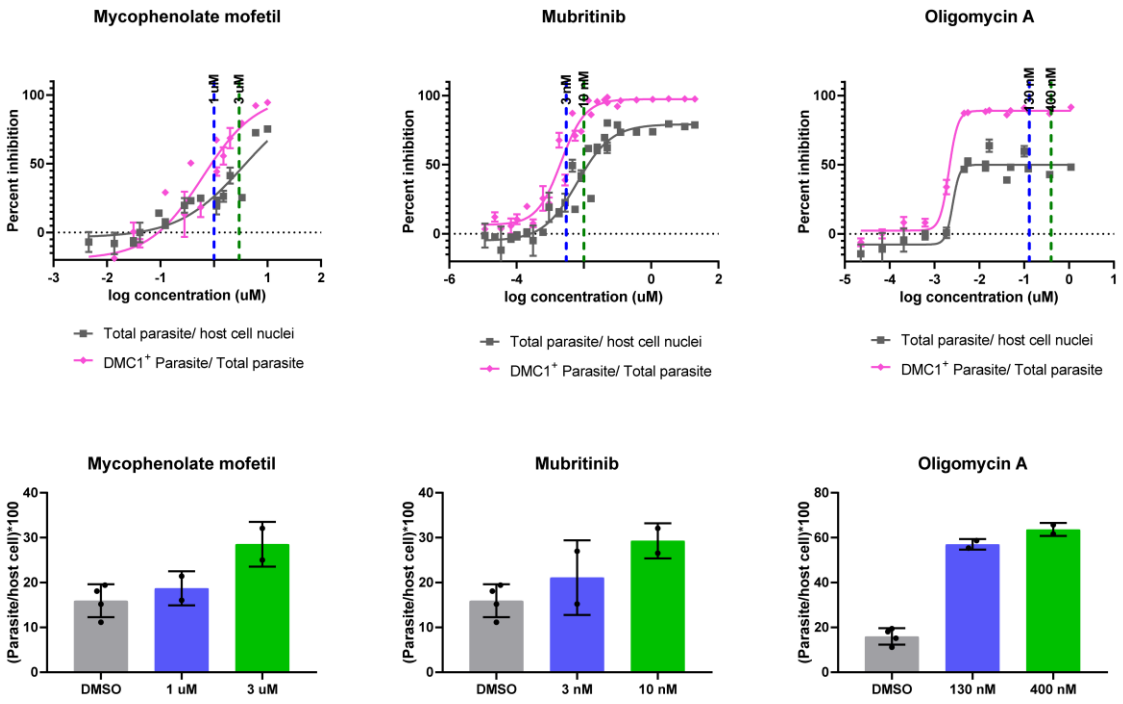

Figure 3: Parasite numbers after 7 days post infection with differentiation inhibitor treatments.

(Top) Dose response curves of total parasite and DMC1+ parasite percent inhibition compared to control, determined from drug addition at 3 hpi and imaging at 72 hpi. Concentrations selected for prolonged (7 days) assays are indicated by the dotted vertical lines. (Bottom) Three differentiation inhibitors were added to *C. parvum* infected HCT8 culture at 3 hours post infection at indicated concentrations. Wells were fixed and imaged at 7 days post infection. *Vicia villosa* lectin (VVL) was used to count parasite number and Hoechst staining was used to count host cell nuclei number. Each point indicates data from replicate wells.

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