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Transcript Diversity In The Protozoan Parasite Toxoplasma Gondii

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Transcript Diversity In The Protozoan Parasite Toxoplasma Gondii

Abstract
Technological advances have made possible to sequence RNA transcripts at unprecedented depth, enabling deep profiling of abundance and diversity under a variety of conditions. Such information permits refinement of draft genome annotation originally generated in the absence of transcript coverage data, and provides new insights into organismal biology and regulatory mechanisms. This dissertation provides an extensive analysis of mRNA-seq data from the obligate intracellular protozoan parasite Toxoplasma gondii, a ubiquitous pathogen of humans and other vertebrates. We produced and sequenced 24 strand-specific RNA libraries from several parasite strains and developmental stages, and examined these in conjunction with 45 additional mRNA-seq libraries produced by other groups.

The current reference genome annotation for T. gondii, generated using de novo methods informed by cDNA sequencing prior to mRNA-seq, identifies ~8300 protein-coding genes, fragmented by ~40K introns. Untranslated regions are incompletely defined, few alternatively-spliced transcripts are described, and non-coding transcripts remain largely unexplored. mRNA-seq datasets presented in this dissertation define a total of 2.7M introns, most observed at vanishingly low abundance. Using current annotation to define parameters minimizing false discovery yields ~60K likely splice junctions. Comparing the frequency of intron-spanning reads to the abundance of transcripts to which introns belong provides a reliable metric for estimating intron excision, readily distinguishing introns that are (i) universally used, (ii) alternatively-spliced, or (iii) likely insignificant. Genome-wide analysis suggests ~3000 annotated introns that should be deleted from the reference genome, ~1400 to be added as alternative isoforms, ~3100 as additions to existing annotation (often within UTRs) and ~3400 associated with novel transcripts. Transcriptomic expression is consistent with biological and phenotypic variation across the complex parasite life cycle, including undescribed differences in gene expression during intracellular tachyzoite replication. Strong circumstantial evidence also suggests that IncRNAs may play an important role in regulating stage-specific expression during sexual differentiation and sporogony. These results provide the basis for revising the reference T. gondii genome annotation available at ToxoDB.org and GenBank. Strategies developed in this dissertation also provide the basis for defining annotation criteria for other species, including related parasites responsible for malaria and conceivably other eukaryotes as well.

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TRANSCRIPT DIVERSITY IN THE PROTOZOAN PARASITE

TOXOPLASMA GONDI

María Alejandra Díaz-Miranda

A DISSERTATION

in

Biology

Presented to the Faculties of the University of Pennsylvania

in

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Degree of Doctor of Philosophy

2017

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TRANSCRIPT DIVERSITY IN THE PROTOZOA PARASITE

TOXOPLASMA GONDII

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who always makes me laugh and shares with me her passions of movies and new and interesting ideas about the world. And then Leo my husband and partner in life, who I met thanks to science, has seen me in my good and not so good days and has constantly supported me with his time and love. This accomplishment is yours too.
ABSTRACT

TRANSCRIPT DIVERSITY IN THE PROTOZOAN PARASITE TOXOPLASMA GONDII

María Alejandra Díaz-Miranda
David S Roos

Technological advances have made possible to sequence RNA transcripts at unprecedented depth, enabling deep profiling of abundance and diversity under a variety of conditions. Such information permits refinement of draft genome annotation originally generated in the absence of transcript coverage data, and provides new insights into organismal biology and regulatory mechanisms. This dissertation provides an extensive analysis of mRNA-seq data from the obligate intracellular protozoan parasite *Toxoplasma gondii*, a ubiquitous pathogen of humans and other vertebrates. We produced and sequenced 24 strand-specific RNA libraries from several parasite strains and developmental stages, and examined these in conjunction with 45 additional mRNA-seq libraries produced by other groups.

The current reference genome annotation for *T. gondii*, generated using de novo methods informed by cDNA sequencing prior to mRNA-seq, identifies ~8300 protein-coding genes, fragmented by ~40K introns. Untranslated regions are incompletely defined, few alternatively-spliced transcripts are described, and non-coding transcripts remain largely unexplored. mRNA-seq datasets presented in this dissertation define a total of 2.7M introns, most observed at vanishingly low abundance. Using current annotation to define parameters minimizing false discovery yields ~60K likely splice
junctions. Comparing the frequency of intron-spanning reads to the abundance of transcripts to which introns belong provides a reliable metric for estimating intron excision, readily distinguishing introns that are (i) universally used, (ii) alternatively-spliced, or (iii) likely insignificant. Genome-wide analysis suggests ~3000 annotated introns that should be deleted from the reference genome, ~1400 to be added as alternative isoforms, ~3100 as additions to existing annotation (often within UTRs) and ~3400 associated with novel transcripts. Transcriptomic expression is consistent with biological and phenotypic variation across the complex parasite life cycle, including undescribed differences in gene expression during intracellular tachyzoite replication. Strong circumstantial evidence also suggests that lncRNAs may play an important role in regulating stage-specific expression during sexual differentiation and sporogony. These results provide the basis for revising the reference *T. gondii* genome annotation available at ToxoDB.org and GenBank. Strategies developed in this dissertation also provide the basis for defining annotation criteria for other species, including related parasites responsible for malaria and conceivably other eukaryotes as well.
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CHAPTER 1: INTRODUCTION & OVERVIEW

The promise of genomics to improve understanding of biology

It has been known for decades that much of the genetic potential of organisms, as encoded in their DNA, is put into practice through the action of proteins, which carry out a wide variety of structural and biochemical functions. This critical conversion is mediated by DNA transcription into messenger RNA (mRNA), and mRNA translation into protein … the ‘Central Dogma’ of molecular biology. More recently, we have come to appreciate that RNA molecules can also carry out a wide variety of regulatory functions, including the modulation of transcription, translation, and mRNA stability (Turner and Morris 2010; Raina and Ibba 2014; Radhakrishnan and Green 2016).

New technologies for nucleic acid library construction and sequencing allow us to profile the entire repertoire of organismal transcriptomes, under a variety of conditions, making it possible to improve our understanding of organismal biology by analyzing the abundance of mRNA and other RNA molecules. Technological developments have also made it possible to conduct similarly comprehensive analysis of other genomic datasets: chromatin modifications, protein composition, etc. The high degree of resolution made possible by these techniques reveals many previously unrecognized RNAs, posing a challenge: how to interpret this vast wealth of information? What is biologically meaningful, and what is meaningless noise?

The goal of this dissertation is to develop methods for extracting maximal information from modern transcriptomic datasets. How can we exploit mRNA transcriptomes to better define gene structures? What is the diversity of other RNA molecules? What role(s) do these RNAs play in regulating gene expression?
My dissertation research focuses on the protozoan parasite *Toxoplasma gondii*, an experimentally accessible human pathogen. Because *T. gondii* displays many of the molecular features observed in other eukaryotic species, it is likely that these methods will be broadly applicable to other organisms as well.

**Eukaryotic gene expression**

While the central dogma of molecular biology outlines the flow of information from DNA to RNA to protein, there are many twists to this basic story. For example, many RNAs – snRNAs, rRNA, tRNAs, and other small and long non-coding RNAs – are never translated into proteins. Instead, they perform biological functions by interacting with or recruiting protein complexes for specific functions. Because RNA is typically single stranded, it can fold onto itself by base-pairing complementary nucleotides within its sequence to create complex three-dimensional shapes capable of performing structural and catalytic functions (Li, Zheng, Ryvkin, et al. 2012; Li, Zheng, Vandivier, et al. 2012; Incarnato and Oliviero 2016). Modern genomic scale projects have revealed that up to three quarters of the human genome is transcribed in one or more cells ... but only half of these transcripts appear to have protein coding potential (Djebali et al. 2012; Harrow et al. 2012). Which of these transcripts play functional roles remains an open question. Understanding transcript expression patterns, how gene expression is regulated, and the biological significance of previously uncharacterized transcripts, is likely to help us understand how cells and organisms alter their behavior in response to external signals.

One common mechanism of transcriptional gene regulation in eukaryotes involves *trans*-acting proteins that recognize and bind to *cis* regulatory sequences in DNA (promoters), typically upstream of the transcription start site. For example, eukaryotic
promoters often include a short sequence of T and A nucleotides \( \sim 25 \) nucleotides upstream of the transcription initiation site, that is bound by the TATA-binding protein. When transcription factors bind to a promoter, they recruit and/or modulate the activity of RNA polymerase on DNA (Todeschini, Georges, and Veitia 2014). After being brought to the promoter region by transcription factors, RNA polymerase starts synthesizing short fragments of RNA until it undergoes a conformational change, brought about by transcription factor-mediated phosphorylation of its C-terminal domain, thereby strengthening binding to DNA and forcing transcription factor dissociation (Wong, Jin, and Struhl 2014; Bowman and Kelly 2014). Cooperation between RNA polymerase and other proteins helps to initiate transcription. For example, activator proteins recognize DNA regulatory regions (enhancers), usually upstream of promoters, helping to recruit transcriptional machinery to the transcriptional start site via mediator proteins that communicate with the polymerase, favoring transcription (Plaschka et al. 2015).

Other biological structures may also affect transcription, including the histone complexes that help to organize the extremely long DNA molecules of eukaryotic chromosomes. Nucleosomal positioning with respect to promoters may impact transcription, as does how tightly DNA is wrapped around the nucleosome, which is determined by histone modifications (Gissot et al. 2007; Croken, Nardelli, and Kim 2013). Histone methylation, acetylation, and other modifications may block polymerase access (Todeschini, Georges, and Veitia 2014) or other components of the transcriptional machinery.
**Transcript processing**

Some non-coding RNAs are known to recruit chromatin-remodeling proteins to specific regions of the genome, modulating the addition of active or repressive marks onto histones (Rinn et al. 2007; Chu et al. 2015) and could also regulate the accessibility of the transcription machinery. Once transcription starts, elongation factors associate with the RNA polymerase and allow it to transcribe for long distances without dissociating from the DNA.

In eukaryotes, primary transcripts are typically processed co-transcriptionally, at the same time that RNA polymerase synthesizes the new transcript. This is possible thanks to the ability of RNA polymerase to change conformations after phosphorylation of its C-terminal domain, exposing other binding sites for multiple proteins that modify the structure of the RNA transcript as it is transcribed. Major modifications to RNA transcripts include the addition of a 5’ ‘cap’, addition of polyadenine nucleotides in its 3’ tail and removal of intervening sequences (introns) by the spliceosomal machinery. Terminal modifications act as protective measures providing transcript stability until translation is set to occur, and may be recognized by the translational machinery.

In contrast, intron excision affects the sequence of the mature mRNA, and often how it is translated into protein. The spliceosome, formed by small nuclear ribonucleoproteins (snRNPs) and auxiliary factors, recognizes specific dinucleotide sequences upstream and downstream of introns (donor and acceptor splice sites), as well as a branch site within intron boundaries, and it performs two trans-esterification reactions between the recognized sequences to excise introns from mRNA transcripts. Spliceosome recognition specificity drives alternative splicing of introns, choosing from
among the various combinations of strong and weak donor and acceptor splice sites within a transcriptional unit (Kornblihtt et al. 2013), providing yet another level of regulation that may yield mature mRNA variants (Wang et al. 2008).

Alternative splicing, based on differential use of splice donors and/or acceptors, may alter exon boundaries, include or exclude exons (exon skipping), or read through introns (intron retention). Because differential splicing affects internal mRNA nucleotide sequences, it may also affect sequence in any resulting translation products, particularly as nucleotide sequences are read in sets of three during translation into protein, so the addition or subtraction of nucleotides may shift the reading frame, altering all downstream protein coding.

Mature mRNA transcripts may also be regulated at various levels, including RNA turnover mediated by degrading enzymes (Houseley and Tollervey 2009), and export from the nucleus where mRNAs are formed to the cytoplasm where translation occurs. Finally, it is also important to note that functional gene expression is also regulated at the level of protein translation and stability. Because of the relative ease of RNA transcript analysis using modern molecular biological techniques, however, steady-state transcript abundance is commonly used as surrogate for measuring functional gene expression.

Eukaryotic genomics, in the era of new sequencing technologies

Although the importance of DNA and RNA in protein production has been understood for more than 50 years, until the advent of high-throughput sequencing methods, this process could only be studied at the level of individual genes, mRNA, or proteins. Modern nucleic acid sequencing technologies make it possible to sequence billions of
nucleotides per day, enabling complete genome sequencing for essentially any organism, even for eukaryotes, whose genomes range from \(\sim 10^7\)-\(10^{11}\) bp.

The sequencing depth reached with modern sequencing technologies gives a near-complete snapshot of transcriptomes with a much higher dynamic range of expression levels and at a base-pair level resolution, in contrast to microarray technologies. However, the increase in depth in several NGS technologies, such as Illumina, SOLiD and 454, is possible thanks to the short length of reads that are sequenced (35-500bp). Short reads are assembled back into full-length transcripts by either \textit{de novo} assembly, based on a reference genome (\textit{ab initio} assembly) or with a combined approach merging these two strategies. The assembly process is not trivial, as it requires large memories in computing systems to assemble large quantities of short reads and also the ability to correctly assign sequencing reads to specific variants of the same transcript in the case of \textit{de novo} assembly. Sequencing reads from both ends of cDNA fragments (100-250bp – paired end protocol) and joining the overlapping reads to form a longer read can help solve the problem posed by short reads. This strategy, however, will not solve assembly difficulties if the paired end reads don’t overlap. An even better approach to avoid assembly problems is to sequence single RNA molecules or large fragments of near-complete cDNAs, as proposed by Helicos and Pacific Biosciences technologies. These technologies also have PCR amplification-free protocols, which usually results in better sequencing coverage of high GC regions, although their sequencing error rates are higher than those from short read technologies. An ideal strategy to study the transcriptome would be to combine sequencing results from different technologies as each of them have advantages and difficulties (Martin and Wang 2011).
The exploration of gene function and expression often begins with analysis of DNA sequence elements, along with attempts to define the most probable gene structure within a DNA sequence. *Ab initio* gene finders rely on recognizing structural features on genes that are extracted from the genomic sequence alone. Prokaryotic genome analysis is now relatively straightforward, for a variety of reasons. In addition to their relatively small genome size (typically $10^6$-$10^7$ bp), prokaryotic promoter sequences are often highly conserved, enabling systematic identification. Further, the lack of intervening sequences fragmenting protein coding genes enables protein products to be predicted by simple translation of long open reading frames (ORFs) using the universal genetic code. In contrast, the promoters and regulatory sequences for most eukaryotic genes cannot currently be defined by specific sequence motifs. And splicing of primary mRNA transcripts produces protein coding regions or exons that are joined by non-coding regions or introns, greatly complicating prediction of complete coding sequences.

Sequencing technology may also be used to determine mRNA sequences after first converting RNA to DNA via the action of reverse transcriptase, and from the outset has been used to sequence individual clones mRNAs, or fragments of randomly-selected mRNAs (expressed sequence tags; ESTs). Because transcript abundance can differ by several orders of magnitude, however, comprehensive cataloging of transcripts initially relied upon hybridization to arrays constructed based on synthetic oligonucleotides, manufactured based on *ab initio* predictions of gene sequences. While immensely valuable for assessing gene-level transcript abundance, such methods are compromised by the inaccuracy of *ab initio* gene predictions, and usually unable to distinguish between alternatively-spliced transcripts.
With the continued increase in sequencing capacity (and decline in cost), it has recently become possible to consider sequencing at sufficient depth to effectively profile essentially all of RNAs in a cell or tissue. This strategy offers several advantages over array-based expression profiling. Because it does not depend on the accuracy of gene model predictions, it provides an assumption-free method for identifying transcripts, regardless of the polymerase used, transcript processing, or their potential to encode proteins. Further, deep sequencing permits identification of alternative transcription products from the same gene. Mapping mature mRNAs back to the reference genome provides direct evidence of alternative transcript initiation, termination, and/or splicing, providing the basis for improved gene-finding algorithms (de novo predictions), guided by mRNA sequences and other lines of evidence (DNA accessibility based on histone positioning, chromatin modification or nuclease sensitivity; transcription factor binding; etc). Methods have also been devised to address other concerns, such as the use of ribosomal profiling to identify transcripts that are actually loaded onto ribosomes, rather than steady-state mRNA levels that fail to take into consideration such important factors as transcript translatability or stability.

Integration of additional extrinsic features into gene finder algorithms permits better definition of gene models, including identification of alternatively spliced transcripts, definition of non-coding regions within genes or UTRs and identification of previously unannotated genes, including non-coding RNA transcripts. The challenge raised by this wealth of data, however, is how to distinguish between biologically important results, and rare events unlikely to manifest themselves in any significant way. For example, the first ab initio annotation of the human genome predicted approximately 30 thousand genes, but pilot projects designed to explore and annotate transcript diversity in detail suggests
up two 200 thousand distinct transcripts (Djebali et al. 2012; Harrow et al. 2012). Are these all functionally significant? The goal of this thesis is to exploit a model eukaryotic system, the protozoan parasite *Toxoplasma gondii*, to develop methods for improving the accuracy of genome annotation as well as applying this knowledge to enhance our understanding of organismal biology.

**Biology of the protozoan parasite *Toxoplasma gondii***

*Toxoplasma gondii* is a unicellular eukaryotic parasite with interesting and clinically important biology. This species’ developmental cycle encompasses both sexual and asexual stages, in distinct environments (Figure 1), presumably requiring distinct patterns of gene expression. Asexual *T. gondii* ‘tachyzoites’ are haploid obligate intracellular parasites, capable of invading any nucleated cell, in any warm-blooded vertebrate. Within the infected cell, tachyzoites establish a specialized ‘parasitophorous vacuole’ within which they replicate clonally, doubling every 8-16 hours until they lyse the infected cell 2-3 days later, releasing new tachyzoites that can infect neighboring cells. All pathogenesis by *T. gondii* (and the many thousands of other parasites in the phylum *Apicomplexa*) is directly attributable to rapid proliferation. Toxoplasmosis in humans is characterized by tissue damage, retinal degeneration, encephalitis, *etc.* In the absence of an effective immune response, the consequences can be fatal.

Tachyzoites are highly promiscuous, and readily able to cross epithelial barriers, including the blood/brain barrier and the placenta. During pregnancy, a primary infection in the mother can be transmitted to the developing fetus, leading to fetal abortion or severe congenital abnormalities. The underdeveloped immune system of the fetus is
unable to recognize and efficiently attack the rapidly dividing tachyzoites, leading to extensive tissue destruction.

In healthy adults, the immune system typically eliminates tachyzoites, but a subset of parasites differentiate into a slowly replicating ‘bradyzoite’ form that is poorly recognized by the immune system, and which can persist within specialized tissues cysts for the life of the host. Latent bradyzoite cysts periodically recrudesce to produce acutely lytic tachyzoites, providing a natural boost to the anti-\textit{T. gondii} immune response … but also threatening immunocompromised individuals, including HIV/AIDS sufferers, or patients immunosuppressed for cancer chemotherapy or transplantation. Bradyzoite tissue cysts are particularly common in muscle tissues and the central nervous system, probably because they are able to persist in these locations for long periods of time as the immune response ramps up. Carnivory is probably the most common source of human infection, as bradyzoite cysts can be transmitted by ingestion of infected tissues.
Humans and other vertebrates become infected with *T. gondii* through the ingestion (or injection) of oocysts from the environment (contaminated soil or water) or tissue cysts from contaminated meat. Excystation is triggered by the acidic environment of the stomach, leading to the emergence of sporozoites or bradyzoites within the small intestine. These motile forms infect the intestinal epithelium and differentiate to form tachyzoites, which replicate rapidly, ultimately lysing the infected cell and invading neighboring cells and tissues throughout the body. This lytic cycle can continue indefinitely, and is responsible for all parasite pathogenesis. A small fraction of tachyzoites differentiate into tissue cysts, perhaps in response to immune attack, containing slow-growing bradyzoites, enclosed within a thin proteoglycan wall impervious to immune attack. Within the intestinal epithelium of felines (only), parasites undergo sexual differentiation to produce micro- and macro-gametes, which fuse to produce an oocyst that is released into the intestinal lumen and excreted in the feces. Upon exposure to oxygen and ambient temperature in the environment, these diploid oocysts undergo rapid meiotic reduction to produce haploid sporozoites.

The sexual stages of *T. gondii* only develop in feline intestinal epithelium, typically after ingestion of an infected mouse. Why sexual development doesn’t occur in other...
animals is unknown, but is typical of the phylum Apicomplexa. For example, Neospora parasites can also infect many species, but only replicate sexually in dogs. The Plasmodium parasites that cause malaria only undergo sexual differentiation in anopheline mosquitoes. In the feline intestinal epithelium, Toxoplasma tachyzoites differentiate into micro- and macro-gametocytes (analogous to sperm and eggs), and fuse to produce diploid oocysts that are shed in cat’s feces. Upon exposure to ambient oxygen, meiotic reduction produces four haploid sporozoites, still encapsulated within the oocyst wall, but ready to start a new asexual cycle when the sporulated oocyst is ingested by the next host via contaminated soil or water. Sporulated oocysts remain viable in the environment for decades.

Once ingested by an uninfected (immunologically naive) host, bradyzoite tissue cysts or sporulated oocysts excyst in the gut, initiating a new round of invasion and asexual replication and completing the parasite life cycle. The ability to be transmitted via contaminated water or food (meat harboring bradyzoite tissue cysts, or vegetables grown in contaminated soil) probably accounts for the ubiquitous nature of this parasite. Approximately one third of the global human population is estimated to be infected with this parasite (Blader et al. 2015).

Although Toxoplasma is a diploid organism, capable of sexual reproduction, note that only haploid forms replicate. As a consequence, the successful progeny of sexual recombination (in cats) yields clonal parasites capable of rapid proliferation. The population biology of T. gondii is characterized by such clonal rockets, transmitted either via carnivory, or through selfing in cats (individual T. gondii zoites are totipotent, able to produce both micro- and macro-gametes). For example, while virtually all parasite
isolates show some evidence of relatively recent sexual recombination, most isolated from North America and Europe belong to just a few closely-related lineages (Howe and Sibley 1995; Khan et al. 2011; Lorenzi et al. 2016).

**Experimental accessibility of Toxoplasma gondii**

Consistent with their promiscuous nature, tachyzoite parasites can be cultivated indefinitely in vitro, in either transformed cell lines (HeLa, CHO, LM, MDBK, Vero, 3T3, etc) or primary cells (foreskin fibroblasts, astrocytes, macrophages, etc). Various stress treatments (oxidative stress, alkali shock, etc) can be used to induce bradyzoite differentiation in vitro. Moreover, infection of mice provides an in vivo model that closely mimics human disease. Sexual differentiation has not been reproducibly observed in vitro, but experimental infection of cats is a reliable (but expensive) method for carrying out traditional genetic crosses. The resulting progeny display typical Mendelian inheritance: four haploid sporozoites are produced from a diploid oocyst after meiotic division (Pfefferkorn, Pfefferkorn, and Colby, 1977; Dubey, Lindsay, and Speer 1998). Because only haploid forms replicate (see above), it is not possible to assess heterozygote phenotypes, but this limitation can be partially addressed by molecular genetic manipulation (see below).

An extensive experimental toolkit is available for exploring T. gondii molecular and cellular biology, including transfection vectors, strategies for insertional mutagenesis, selectable markers, conditional and inducible expression, knockout and gene manipulation systems (including the CRISPR technology), localization of organelles and/or genes by tagging with fluorescent probes, etc (Donald and Roos 1993; Sibley, Messina, and Niesman 1994; Roos et al. 1995; Soldati 1996; Belperron et al. 2001; Bradley, Li, and
Moreover, as a member of the protozoan phylum apicomplexan, including many other parasites of clinical and economic significance (*Plasmodium* species are responsible for malaria, *Cryptosporidium* is a leading cause of diarrheal disease in infants, *Eimeria* is the leading infectious disease concern in the poultry industry, *Babesia*, *Neospora* and *Theileria* are important cattle pathogens, *etc*), many of the experimental tools and insights gleaned from *Toxoplasma* have proved to be applicable to other parasites. For example, transfection systems developed for *Toxoplasma* provided the basis for transfection of *Plasmodium*, and insights into mechanisms of drug resistance, the process of cell invasion, the biology of the apicoplast (see below) and some aspects of host-parasite interactions have proved to be of general interest (Kim and Weiss 2004; Blader et al. 2015; McFadden and Yeh 2017).

**Toxoplasma gondii cell and molecular biology**

*Toxoplasma* harbors a typical complement of eukaryotic organelles, including a typical eukaryotic nucleus, cytoskeletal elements, complex endomembrane system, and mitochondria. Some organelles present distinctive features, however. For example, in contrast to the open mitosis observed in metazoan systems, the nuclear envelope remains intact throughout mitosis (closed mitosis, as observed in most unicellular eukaryotes). The nuclear envelope plays an important role in chromosome segregation, as it organizes the centrosome, mitotic spindle, and chromosomal centromeres to form the centrocone (Brooks et al. 2011; Farrell and Gubbels 2014). *Toxoplasma* also harbors various apicomplexan specific organelles, including the apicoplast (a non-
photosynthetic plastid acquired by secondary endosymbiosis of a eukaryotic alga, and retained as an essential organelle; (Köhler et al. 1997; Ralph et al. 2004), apical secretory organelles (micronemes and rhoptries), and the apical polar ring and conoid (Graindorge et al. 2016). These organelles constitute the apical complex, for which the phylum Apicomplexa is named. Secretory organelles play distinct roles in key aspects of the life cycle, including host cell attachment, invasion and egress (Besteiro et al. 2009; Huynh, Boulanger, and Carruthers 2014; Roiko, Svezhova, and Carruthers 2014; Huynh and Carruthers 2016), establishment and maintenance of the parasitophorous vacuole, and intracellular survival and interaction with the host cell (Saeij et al. 2007; Fentress et al. 2010; Reese et al. 2011; Fentress et al. 2012; Fleckenstein et al. 2012; Fox et al. 2016; Leroux et al. 2015).

At ~ 65 Mb (14 chromosomes), the Toxoplasma DNA genome is significantly smaller than animal, plant, and many other parasite genomes, but much larger than prokaryotic genomes. Genes are not arranged in operons, as in prokaryotic microbes, and transcription is mediated by typical eukaryotic polymerases and part of the basal transcriptional machinery, recruited by some conserved general transcription factors (TFIID, E, F, H, etc), under the influence of promoters, enhancers and chromatin marks similar to those found in other eukaryotes (Meissner and Soldati 2005; Sullivan et al. 2013). Apicomplexan transcription factors are dominated by AP2-integrase domain-containing proteins (Balaji et al. 2005), which are among the 20 most abundant Pfam domains in tissue-cyst forming coccidian parasites like T. gondii (Lorenzi et al. 2016).

Primary T. gondii mRNAs are capped and polyadenylated – although there is also evidence of non-polyadenylated transcripts – and contain numerous cis-introns, but no
known trans-splicing. Introns are excised using standard eukaryotic spliceosomal machinery, as human nuclear extracts can properly splice *T. gondii* primary transcripts, and vice versa. These features are important for both ab initio and de novo gene finders, as well-characterized human splice signals can be incorporated as intrinsic features during gene detection.

*Toxoplasma* genes were first cloned in the 1980s and sequencing of random expressed sequence tags (ESTs) in the 1990s defined several thousand additional genes expressed in tachyzoite parasites (Ajioka et al. 1998). Northern blotting, qPCR and analysis of EST frequency provided the first glimpse of transcript abundance, including identification of related transcripts suggesting alternative splicing (Donald et al. 1996; Chaudhary et al. 2005).

A first draft of the *T. gondii* reference genome completed in 2002 (shortly after completion of the human genome sequence) and assembled EST clusters mapped to the genome defined many gene models used to train ab initio and de novo gene finders predicting a total of approximately 8000 genes with protein coding potential. With the availability of annotated gene models, microarrays were designed to assess the transcriptional levels for all predicted genes (Bahl et al. 2010). This platform has been used by the global *T. gondii* research community to define stage- and strain-specific gene expression (see ToxoDB.org; Kissinger et al. 2003). As noted above, however, assessment of expression levels in microarray experiments poses several limitations. Background fluorescence signal corrections, typically excludes analysis of weakly expressed transcripts. Furthermore, since probe design is restricted to correct gene number estimation and requires preexisting knowledge of gene structure, microarray technology is
not well-suited to identifying errors in the present gene annotation, alternative transcripts, or unannotated genes. In contrast, RNA-seq technologies are able to profile all transcripts. These characteristics render RNA-seq technology suitable for evaluating transcriptome expression and improving the quality and accuracy of genome annotation.

Overview of this dissertation

By enabling comprehensive interrogation of organismal genomes, genomic-scale datasets are becoming an increasingly important tool in biological and biomedical research. This wealth of information holds promise for improved genome analysis, and new insights into the molecular mechanisms of many biological processes. The abundance of data also creates a serious challenge: How to better identify and interpret relevant information from these datasets? How do we discriminate biological significance and incorporate this information into decision-making? More specifically, is my gene-of-interest expressed? Is it correctly annotated, in all its forms?

The goal of this thesis is to develop analytic methods that permit discrimination of significance among genome-scale datasets, particularly transcriptome data derived from RNA-seq experiments. In pursuit of this goal, we have focused on the genome of *Toxoplasma gondii*, comparing current gene annotation to transcriptional evidence derived from RNA-seq experiments across all life cycle stages in order to identify errors in gene annotation, unannotated genes, and alternatively spliced transcripts.

As briefly described above, *T. gondii* is a unicellular eukaryotic parasite capable of causing disease in immunocompromised humans and animals, and a well studied organism, with a near complete genome sequence and a myriad of experimental tools. We constructed and sequenced 24 strand-specific RNA-seq libraries intended to explore
transcriptional variation across the intracellular lytic tachyzoite stage of the life cycle, and examined these together with 45 additional sequenced libraries profiling transcription in other life cycle stages. All of these datasets are now available on line at ToxoDB.org, and deposition in GEO is in progress.

These datasets have been used, in conjunction with other functional genomic information where appropriate, to explore the feasibility of identifying stage-specific markers based on transcriptional profiling data, and to improve the status of genome annotation, including untranslated regions, previously unrecognized exons and alternatively spliced transcripts, and novel transcripts, including long non-coding RNAs (IncRNAs). Throughout this dissertation research, I have attempted to emphasize methods that should be broadly applicable to other eukaryotic microbes for which similar datasets are available.
CHAPTER 2: USING SEQUENCING TECHNOLOGIES (AND OTHER LARGE-SCALE DATASETS) TO ASSESS AND IMPROVE GENOMEANNOTATION IN TOXOPLASMA GONDII (Adapted From Paper)

New sequencing technologies have made many species accessible to genomic-scale analysis, raising the challenge of how to integrate such information from various sources, discriminate biological significance, and make these results accessible to diverse end-user communities. We have exploited strand-specific RNA-seq analysis to profile the transcriptome of human host cells infected with the protozoan parasite Toxoplasma gondii, a prominent eukaryotic microbial pathogen responsible for disease during congenital infection and in immunosuppressed individuals (Tenter, Heckeroth, and Weiss 2000).

At ~65 Mb in length, the T. gondii genome is relatively compact (Lorenzi et al. 2016), but harbors most of the complexity described for other eukaryotic genomes, including ~8300 protein coding genes, ranging in length from <1 to >60 kb (ave ~4.8 kb), and fragmented by introns (range 0-60+; ave ~5.8) that follow consensus eukaryotic sequence constraints (primary T. gondii transcripts are properly spliced by human nuclear extracts). Extensive population genetic and functional genomic datasets are available for T. gondii, including additional RNA-seq data and other transcriptional profiles for various strains and developmental stages, proteomics data, chromatin marks, etc (ToxoDB.org; Gajria et al. 2008). As noted above (Chapter 1), numerous tools are also available for experimental manipulation of T. gondii in the laboratory (Roos et al. 1995; Sibley et al. 2002; Kim and Weiss 2004; Meissner et al. 2007; Sidik et al. 2016). We have generated strand-specific RNA-seq datasets for various T. gondii tachyzoite
strains, at multiple time points during their 48 hr intracellular in vitro replicative cycle, and analyzed these in parallel with datasets from other life cycle stages, to assess the accuracy of current genome annotation, identify new genes (including alternatively spliced transcripts), and assess stage-specific transcript expression and regulation (Chapter 3).

Methods

Parasite cultures, RNA isolation, RNA library construction and sequencing

*T. gondii* tachyzoites from four different strains (ME49, VEG, RH, GT1), were maintained by serial passage in human foreskin fibroblast (HFF) monolayers as previously described (Roos et al. 1995), infecting confluent monolayers with ~$10^7$ tachyzoites. For time course experiments, parasites were propagated in Vero cells (*Cercopithecus aethiops*) for two passages immediately before the infection of HFFs for RNA isolation, to avoid inadvertent contamination with human material from the previous infectious cycle. After media removal, cell monolayers were scraped in 700ul of Qiazol, and RNA isolated from cell lysates using the Qiagen miRNEasy mini kit, according to the manufacturer’s instructions. Six biological replicates were collected per strain and timepoint, and checked for RNA quality using a BioAnalyzer (Agilent). Strain M4 bradyzoites, strain CZ-H3 enterocytes (gametocytes), and strain M4 oocysts were prepared and RNA isolated as previously described (Buchholz et al. 2011; Fritz, Buchholz, et al. 2012; Juránková et al. 2013; Basso et al. 2013; Hehl et al. 2015). Biological replicates were pooled, and total and polyA+ selected RNA used to construct strand-specific mRNA (and in some cases small non-coding RNA) libraries as previously described (Li, Zheng, Vandivier, et al. 2012; Elliott et al. 2013), and sequenced on Illumina Hi-Seq 2000 (see
Table 1 for total number of reads per library). All RNA-seq data described in this study are available from the *Toxoplasma* Genome Database, at ToxoDB.org (Gajria et al. 2008), along with other RNA-seq datasets and diverse additional information (Fritz, Bowyer, et al. 2012; Minot et al. 2012; Reid et al. 2012; Lorenzi et al. 2016).

Alignment of RNA-seq reads to the *T. gondii* genome: the ToxoDB pipeline for mapping RNA-seq reads

For transcript assembly, RNA-seq reads were initially mapped onto *T. gondii* ME49 genome release 28 using RUM (Grant et al. 2011); subsequent studies used GSNAP (Wu and Nacu 2010) to map to genome version 29. The RUM alignment pipeline takes advantage of the speed of Bowtie (Langmead et al. 2009) to map against both the genome and transcriptome; unmapped reads are then mapped against the genome using Blat (Kent 2002), and Information from all three mappings is then merged. GSNAP was configured to look for both known and novel splicing. Coverage was determined for unique and non-unique alignments (separated by strand when possible). Strand orientation of splicing was determined based on the usage of GT/AG, GC/AG, or AT/AC dinucleotide pairs on the plus strand (or their complements on the minus strand). In cases where strand could not be defined, the program applies a probabilistic splice model to determine orientation (Wu and Nacu 2010). Performance for both RUM and GSNAP tools is comparable to the best RNA-seq alignment tools available at the time this study was completed (Engström et al. 2013).

Algorithm for gene model learning and prediction

Gene model training and predictions were performed using a version of CRAIG (A. Bernal et al. 2007) that integrates RNA-seq data, encoded as features derived from the
mapping of reads to the *T. gondii* genome, as described above. The evidence integration strategy and feature encoding for RNA-seq data have been reported previously (Bernal, Crammer, and Pereira 2012; Bernal and Pereira 2012). ToxoDB v28 gene annotations were used for training, after filtering to exclude genes with evidence of significant alternative splicing (see below). The learned model integrates *ab initio* features such as segment length distributions, with features derived from junction-spanning and coverage reads. We sought completeness in transcript prediction by forcing CRAIG to define at least one transcript model for each non-overlapping transcript junction (putative intron) with read counts >3, and for overlapping junctions, those displaying >20% of the highest support observed in any overlapping junction within the same gene model.

**Assessment of genome annotation, analysis of alternative splicing and visualization**

To assess the quality of the reference *T. gondii* genome annotation, constructed largely based on *ab initio* methods informed by EST sequences from tachyzoite stage parasites only, expression data was retrieved for all predicted introns in 69 RNA-seq samples available in ToxoDB.org (Table 1), including samples from many strains, and most *T. gondii* life cycle stages (see Chapter 1 for a description of the parasite’s asexual and sexual life cycles). This yielded a list of 2,731,523 candidate introns, but many were observed in only one or two samples, or at very low abundance levels in any sample. Introns observed <6 times overall, or with <3 read in any of the 69 samples considered, were excluded from further analysis, leaving a total of 147,715 for in-depth analysis (including 997 previously-annotated introns not satisfying the above criteria).
Table 1. List of T. gondii RNAseq datasets used in this study (ToxoDB release 28)

| Dataset | Strain | Stage | Host | Cond | Breed | RNA | Str Spec | Ins Size | Read Len | Total Reads | FPKM Unique | % | Total SRIs | % |
|---------|--------|-------|------|------|-------|-----|---------|----------|----------|-----------|-------------|-------------|---|------------|---|

Intracellular Tachyzoites – Diverse Strains

| Dataset | Strain | Stage | Host | Cond | Breed | RNA | Str Spec | Ins Size | Read Len | Total Reads | FPKM Unique | % | Total SRIs | % |
|---------|--------|-------|------|------|-------|-----|---------|----------|----------|-----------|-------------|-------------|---|------------|---|

Intracellular Tachyzoites – Developmental Series

| Dataset | Strain | Stage | Host | Cond | Breed | RNA | Str Spec | Ins Size | Read Len | Total Reads | FPKM Unique | % | Total SRIs | % |
|---------|--------|-------|------|------|-------|-----|---------|----------|----------|-----------|-------------|-------------|---|------------|---|

Bradypire development

| Dataset | Strain | Stage | Host | Cond | Breed | RNA | Str Spec | Ins Size | Read Len | Total Reads | FPKM Unique | % | Total SRIs | % |
|---------|--------|-------|------|------|-------|-----|---------|----------|----------|-----------|-------------|-------------|---|------------|---|

Gametocyte development

| Dataset | Strain | Stage | Host | Cond | Breed | RNA | Str Spec | Ins Size | Read Len | Total Reads | FPKM Unique | % | Total SRIs | % |
|---------|--------|-------|------|------|-------|-----|---------|----------|----------|-----------|-------------|-------------|---|------------|---|

Oocyst development

| Dataset | Strain | Stage | Host | Cond | Breed | RNA | Str Spec | Ins Size | Read Len | Total Reads | FPKM Unique | % | Total SRIs | % |
|---------|--------|-------|------|------|-------|-----|---------|----------|----------|-----------|-------------|-------------|---|------------|---|

Other samples

| Dataset | Strain | Stage | Host | Cond | Breed | RNA | Str Spec | Ins Size | Read Len | Total Reads | FPKM Unique | % | Total SRIs | % |

References

1. Lorenz H et al., Nature Communications; 2016 (ref 7)
2. Minour et al., Proc Natl Acad Sci; 2012 (ref 18)
3. Reid et al., PLoS Pathogens; 2012 (19)
4. This study
5. Pittman et al., BMC Genomics; 2014 (20)
6. Fritz HM et al., PLoS; 2012 (17)
7. Yesh UM et al., Natl Acad Sci; 2015 (21)
9. Unpublished; available from ToxoDB.org
Table 1. List of T. gondii RNAseq datasets used in this study (ToxoDB release 28).
Green highlighting indicates 20 high quality samples used define the prevalence of alternative splicing and mechanisms of transcriptional regulation; pink highlighting indicates reasons for exclusion of other samples from the analyses presented in Chapter 2; see text for further discussion.

Because strand-mapping information was not retained in the ToxoDB pipeline implementation of RUM, strandedness was assigned by analyzing five nucleotides up-and downstream of each intron to determine the most probably splice donor and acceptor. Analysis of the abundance distribution of dinucleotides pairs for each intron, showed that (as expected) the most common splice signal (on the plus strand) was 5'-GT/AG-3’, which is >80 times more abundant than any of the other possible 63 intron combinations. 5’-GC/AG-3’ and 5’-GA/AG-3’, were the next most common (enriched 1.8 & 1.4 times, respectively). We therefore further filtered this intron list to include only introns that contained the major splice signal 5’GT/AG3’. This procedure yields a total of 66,104 introns for examination in greater detail.

Once strand was recovered, introns were assigned to gene structures to determine those fully contained within a previously-annotated gene model, those lying fully within intergenic regions in the draft annotation (potential extensions of existing gene models, or associated with previously unrecognized genes), and those overlapping draft gene models. For introns associated with a specific gene, reads spanning that intron should be comparable to reads mapping to the mature transcript. The abundance of intron-spanning reads (ISRs) per million reads in the library (ISRPM) was therefore plotted as a function of the number of reads mapping to the assigned gene, normalized to gene size (FPKM = read fragments per kilobase of transcript, per million total mapped reads).
Preliminary analysis using all 69 RNA-seq samples in Table I revealed poor correlation between intron and gene expression for a some introns, invariably attributable to poor quality samples in which relatively few reads could be mapped to the parasite (or host) genome. These samples were therefore excluded from analysis of splice junction usage, as were samples for which only non-strand-specific reads are available, and samples involving splicing machinery mutants (pink shading in Table 1). Note, however, that data from all these samples remains available in ToxoDB, and were reviewed after the completion of our analysis of high confidence introns.

All further analysis was conducted using a final set of 59,755 introns, from 20 samples representing all parasite life cycle stages, in multiple strains (green shading in Table 1). To analyze the prevalence of alternative splicing, the abundance of each intron was compared to its most abundant alternative(s), if any. Data was visualized using DataGraph 4.1 (Visual Data Tools; Figs 2.4-2.6 & 2.12-2.13).

Results

Transcriptional insights from RNA-seq, applied to Toxoplasma gondii

Prior to the development of high throughput methods for mRNA sequencing, eukaryotic gene finding relied upon on ab initio methods (predicting gene structure based on primary sequence alone), and de novo strategies informed by cDNA sequences from expressed sequence tags (ESTs; Burge and Karlin 1998; Salamov and Solovyev 2000; Wei and Brent 2006). The much greater coverage provided by low cost RNA-seq methods greatly enhances gene model accuracy, however, enabling the identification of previously unrecognized transcripts, refinement of untranslated region (UTR) annotation,
definition of stage- and strain-specific transcripts, recognition of alternative splice junctions, etc (Trapnell et al. 2010). When available, additional genomic-scale datasets (chromatin marks, transcription factor binding sites, protein expression data, etc) can also be exploited further improve the accuracy of gene model prediction (Lamesch et al. 2012).

TgHXGPRT was the first alternatively-spliced gene identified in the protozoan parasite Toxoplasma gondii (Donald et al. 1996), based on the presence or absence of an 'exon skip' polymorphism encoding a 49 amino acid insertion including an acylation motif responsible for protein association with parasite membranes (Chaudhary et al. 2005). The reference model in the official GenBank annotation (and ToxoDB) includes this exon-skip polymorphism as exon III (Fig 2.1 track 1; blue indicates transcription from left-to-right, i.e. on the forward, or top strand). Numerous ESTs map to TgHXGPRT (track 2), confirming both splice variants: exon III is missing from eleven ESTs (HXGPRT-I), but included in six (HXGPRT-II). Western blotting indicates similar relative abundance of HXGPRT-I vs HXGPRT-II at the protein level (Chaudhary et al. 2005). This well-validated example of an alternatively-spliced gene was used as a positive control to define and assess parameters for alternative transcript identification genome-wide.

RNA-seq data provide vastly greater experimental support: average depth for the experiment presented in Fig 2.1 is >700 reads (track 3; \(2^{8.5}\) on the log scale plot shown in track 5). The most common apparent transcript initiation site (in this steady-state analysis) occurs at \(\sim 6,795,950\) (heavy magenta arrow), \(\sim 100\) nt downstream of the annotated 5’ end. In keeping with common conventions from the pre-RNA-seq era, this gene was originally annotated based on the longest, rather than the most abundant
cDNA clone. Log-scale representation reveals the range of 5’ ends identified by RNA-seq, although we cannot exclude the possibility of 5’ exonuclease activity or premature termination during reverse transcription. Most transcripts appear to terminate close to the annotated 3’ end (*filled magenta circle*), although alternative low abundance termination sites are also evident, most prominently ~500 nt downstream (*open magenta circle*).

**Figure 2.1. Reading RNA-seq data.**

The annotated *T. gondii* HXGPRT gene (TgME49_200320), aligned to EST sequences and RNA-seq data (both linear & log coverage plots), including intron-spanning reads (brackets). Note the presence of multiple 5’ ends (at steady-state), likely corresponding to multiple promoters (magenta arrows), of which only the longest and most prominent would permit excision of intron I (which lies within the 5’UTR). EST coverage in intron I was previously misinterpreted as intron read-through variants (Donald et al. 1996). Magenta brackets highlight the well-validated exon skip variant (star) responsible for membrane association of HXGPRT isoform II (Donald et al. 1996; Chaudhary et al. 2005). Multiple (low abundance) 3’ ends are also observed (circles).
Six well-defined exons are clearly identifiable in the RNA-seq coverage plots, corresponding precisely to the annotation TgHXGPRT-II. Exon III (*magenta star*) is less abundant than the other five, however (seen most clearly in the linear representation; track 3), providing evidence of alternative splicing, consistent with ESTs, Northern blotting, protein immunoprecipitation, proteomics and protein structure data ([ToxoDB.org](http://ToxoDB.org) and [Chaudhary et al. 2005](http://Chaudhary et al. 2005)). RNA-seq reads that map across intron junctions (intron-spanning reads; ISRs) are indicated by horizontal brackets in track 4 (pooled information from numerous experiments). *Magenta brackets* highlight introns corresponding to the known HXGPRT exon-skip polymorphism: for the experiment shown (*TgCZ-H3 tachyzoites*), 149 reads could be unequivocally mapped to intron II, 187 reads span intron III, and 180 reads span introns II+III (excising exon III), defining the HXGPRT-I transcript. Pooling all available experimental data (from multiple samples) provides overwhelming support for this exon-skip polymorphism (8573, 11475, and 12785 reads, respectively; ToxoDB release 28).

Intron-spanning reads (ISRs) also identify numerous unannotated introns, but these are significantly less abundant. For example, while intron I is supported by 391 reads in the experiment shown (27K in all studies), an alternative splice donor 112 nt upstream is supported by 7 ISRs and 227 ISRs respectively, and this alternative intron is also supported by EST data; two ISRs (61) support yet another alternative donor 8 nt further upstream. Note that none of these alternatives affects the predicted protein sequence, however, as intron I lies within the 5’ untranslated region (UTR). Alternative ISRs mapping to the HXGPRT coding sequence also seem unlikely to be biologically meaningful, as the most common (87 reads in all studies, but none in the experiment shown) extends
intron IV by 17 nt, which would introduce a translational frame shift and premature termination eliminating most of the phosphoribosyl transferase domain (Pfam00156).

In addition to defining the exon skip polymorphism distinguishing HXGPRT-I & II, the original cDNA clones were interpreted to suggest retention of intron I in some transcripts (Chaudhary et al. 2005). Read coverage within intron I is significantly higher than other introns (~10% exon depth vs <5% for other introns), but careful examination of the RNA-seq data reveals that coverage is non-uniform, displaying gradually increasing depth in the direction of transcription, suggesting alternative promoters (lighter magenta arrows). This interpretation is consistent with both EST evidence and review of the original cDNA clones. Transcript initiation within intron I would of course preclude intron excision.

In sum, using the highly-curated TgHXGPRT gene as a positive control for reanalysis based on RNA-seq data improves the definition of UTRs, confirms exon boundary annotation and a known exon-skip variant, permits identification of additional rare (and probably biologically meaningless) splice variants, and reveals that the previously-described intron retention is more likely attributable to alternative transcript initiation within intron I. Applying such analyses genome-wide offers the prospect of significantly improved gene model definition. For example, as shown in Fig 2.2, previous annotation (ToxoDB release 7.3, produced without the benefit of RNA-seq data) failed to define 5’ UTRs for 5777 of the 8323 protein-coding genes in the reference T. gondii annotation, and 3’ UTRs for 4859 (gray bars). Incorporating RNA-seq information now identifies 7219 5’ UTRs and 7296 3’ UTRs, with a modal 5’ UTR length of ~750 nt, and 3’ UTR length of ~500, similar to HXGPRT, above (blue in Fig 2.2).
Fig 2.3 presents an expanded genomic region, extending upstream of HXGPRT (the right-most gene in this panel), including RNA-seq evidence from both tachyzoite and gametocyte (enteroepithelial) stage parasites (Hehl et al. 2015), along with additional genomic-scale data from chromatin immunoprecipitation studies (Gissot et al. 2007). These experiments support the reference annotation of \textit{TgME49_200310} (immediately to the left of HXGPRT), which is heavily transcribed in tachyzoites (tracks 5,6,8), gametocytes (track 10), and other life cycle stages (not shown). Low abundance unannotated ISRs never exceed 2\% of annotated intron abundance for this gene. Chromatin activation marks (H3K4me3 & H3K9ac; track 1) are consistent with a 242 nt region mediating divergent transcription of \textit{TgME49_200310} & 200320 (HXGPRT).

**Figure 2.2.** Length distribution of annotated UTRs. Lengths of UTRs before (\textit{gray}) and after (\textit{blue}) incorporating RNA-seq data into gene finding algorithms.
Figure 2.3. Strand-specific mRNA-seq reveals a multitude of alternative splice variants and antisense RNAs, including long non-coding RNAs.

Genome browser view of a 22kb region (TgME49_chrVIII: 6778-6800kb) displaying publicly available chromatin marks (H3K4me3 & H3K9ac, track 1), annotated gene models (including HXGPR, at right, track 3), non strand-specific mRNA-seq data (tracks 5,6), and selected strand-specific mRNA-seq data from this study (tracks 7-10). Color intensity in track 4 indicates 10-fold differences in the abundance of junctional reads (dark brown > light brown > orange). Arrows and circles (tracks 8,10) indicate likely 5’ and 3’ termini, respectively; green shadowed transcript (track 10) proposes a corrected gene model; the green box highlights a likely long noncoding RNA (lncRNA). See text for further discussion.

In contrast, RNA-seq data argues for revised annotation of the adjacent region (ChrVIII: 6.778-6.792 Mb). Ab initio gene-finding methods previously suggested a seven-exon transcript (TgME49_000300; track 2), while more recent annotation taking
(limited, non-strand-specific) tachyzoite-stage RNA-seq data into account were used to define two transcripts: TgME49_200300 & 200295 (track 3). Strand-specific sequencing of multiple life cycle stages helps to reconcile these discordant views. In tachyzoite stage parasites (track 8), steady-state RNA levels indicate two 5’ ends, consistent with the above gene models (light green arrows), but provide no support for either exon I or intron I from the first draft annotation (TgME49_000300). In gametocyte stages, however (track 10), transcription initiates far upstream (heavy green arrow), at a position consistent with chromatin modification data (track 1), and similar to the original ab initio predictions. The resulting 17-exon gene model (green shading) encompasses all exons previously suggested by any annotation, along with additional exons revealed by high-depth sequencing (mostly within the 3’UTR). As noted for the HXGPRT gene, while CDS intron excision is generally efficient, relatively high transcript abundance is sometimes observed within UTR introns, due to a combination of alternative transcript initiation, termination, splicing, and/or inefficient intron excision.

Interestingly, while the first exon of this gene (from ~6.790-6.792 Mb) is only observed in gametocyte stage parasites (track 10), this region is heavily transcribed on the opposite (forward, blue) strand in tachyzoites (track 7). The lack of an open reading frame on this strand, and lack of chromatin marks typically associated with active Pol II-mediated mRNA transcription (track 1) suggests that it is a long non-coding RNA (lncRNA). This interpretation is consistent with the low prevalence of introns mapping to this transcript:

Just 39 reads were observed spanning the most abundant intron seen in this experiment (97 in all experiments), at least 4-fold fewer than expected based on transcript
abundance (compare with read coverage troughs corresponding to introns for the HXGPRT gene, for example). This IncRNA is missing in gametocytes, i.e. it’s presence is inversely correlated with transcription on the reverse (red) strand.

**Genome-wide analysis of putative introns, and alternatively splicing**

In order to exploit the utility of RNA-seq data for improving genome annotation, including the identification of stage-specificity, UTRs, alternative splicing, etc, we generated several strand-specific libraries from various parasite strains, at different times after infection of human host cells in vitro (see Methods), and considered the resulting RNA-seq data in parallel with all other T. gondii RNA-seq datasets available in the ToxoDB database (release 28). The 69 samples summarized in Table 1 (above) derive from nine studies involving various parasite strains, mutants, life cycle stages and host species.

A total of $1.1 \times 10^9$ mRNA-seq reads from wild-type parasites map uniquely to the *T. gondii* (not host) genome, and define ~2.7M splice junctions. The vast majority of these correspond to the U2 (GT/GC-AG) or U12 (GA-AG) splice consensus, suggesting genuine spliceosomal origin, but >90% were observed at very low abundance (<6 total reads, or <3 in the most abundant sample), and were not analyzed further. Introns whose strand could not be unambiguously determined were also excluded from this study. Further restricting analysis to strand-specific libraries with >$10^6$ parasite-specific reads leaves a total of 20 samples – 11 of which were generated specifically for this study, (and 16 of which are presented here for the first time). These samples provide support for >97% of the ~40K introns annotated in the current reference genome, in addition to ~20K unannotated introns that might reasonably be considered for inclusion in the reference annotation. Adding back ~1K annotated introns unsupported by RNA-seq
evidence yields a total of 59,755 candidate introns for further analysis (see Supplemental File 1).

Assuming uniform read coverage across all full-length transcripts, unambiguous mapping, and efficient splicing ... and ignoring alternative splicing for the moment, one would expect a linear relationship between the abundance of intron-spanning reads (ISRs) and all reads that map to a transcript, normalized for transcript length (Venables et al. 2008; Katz et al. 2010). All introns were mapped to individual genes or intergenic regions, and intron abundance was plotted as a function of transcript abundance, as shown in Figure 2.4. Intron abundance (ISRPM) is defined for each junction as the number of ISRs spanning that junction, normalized to the total number of ISRs mapped in that experiment (in millions). Transcript abundance (FPKM) is defined for each gene as the number of reads (or read pairs) mapping to that gene, normalized to gene length (in kilobases) and the total number of read (pairs) mapped in that experiment (in millions).

The data in Fig 2.4 are derived from a single experiment on the acutely lytic (tachyzoite) stage of T. gondii strain CZ-H3 (Basso et al. 2013; Juránková et al. 2013). Annotated introns are shown in black, while unannotated introns are shown in gray; unannotated intergenic introns are not displayed, as their FPKM values are not readily defined. Two populations of introns are immediately evident: low abundance introns mapping to genes at various expression levels (shaded area in Fig 2.4), and introns that appear to be efficiently excised (i.e. ISRPM proportional to FPKM, regardless of expression level; diagonal in Fig 2.4). It is clear that the latter are predominantly annotated (black), while the former are predominantly unannotated (gray).
Figure 2.4. The abundance of annotated introns is overwhelmingly correlated with total transcript abundance.

Intron-spanning read abundance (ISRPM) as a function of transcript coverage (FPKM), for annotated introns (black) and novel (unannotated) introns (gray), in *T. gondii* tachyzoites. Just 31K introns are reproducibly observed and map to annotated genes at the expected abundance levels. Most of the introns observed at levels comparable to transcript abundance are annotated, i.e. black dots lie along the diagonal. Magenta dots highlight HXGPRT introns (see Fig 2.1), including (from top to bottom) the invariably-spliced introns IV & V and the slightly less-efficiently excised 5'UTR intron I; alternatively-spliced introns II, III and the intron II-III exon skip isoform; and several low abundance unannotated introns (mostly off-scale).

Assuming the reference annotation provides some approximation of the truth (even if it cannot be considered a gold standard), and that systematic errors in gene model
prediction are not overwhelming, the current annotation can be used to define parameters for intron abundance and splicing efficiency that minimize false discovery (Supplementary Fig S1). As quantified in Table 2A, the majority of introns that are efficiently excised in tachyzoite-stage parasites (ISRPM/FPKM ≥0.2) are annotated (98%), and the majority of annotated introns (81%) are efficiently excised. Conversely, the majority of low abundance introns (ISRPM <1, regardless of FPKM) are not included in the reference annotation (57%), and the majority of unannotated introns are low abundance (88%), even after exclusion of >2.5M very low abundance or poor quality unannotated introns (see above).

Table 2A. Intron abundance & annotation in various samples.

<table>
<thead>
<tr>
<th>Intron Abundance</th>
<th>Excised (ISRPM)</th>
<th>Excised (ISRPM/FPKM)</th>
<th>Unannotated</th>
</tr>
</thead>
<tbody>
<tr>
<td>High (≥10)</td>
<td>10,855</td>
<td>10,855</td>
<td>2,535,138</td>
</tr>
<tr>
<td>Med (1-10)</td>
<td>8,268</td>
<td>8,268</td>
<td>12,805</td>
</tr>
<tr>
<td>Very Low (&lt;0.2)</td>
<td>1,193</td>
<td>1,193</td>
<td>7,325</td>
</tr>
</tbody>
</table>

Table 2B. Alternative splicing in T. gondii.

<table>
<thead>
<tr>
<th>Alternative Intron</th>
<th>Reference (ISRPM)</th>
<th>Alternative (ISRPM ≥1 only)</th>
<th>Unannotated</th>
</tr>
</thead>
<tbody>
<tr>
<td>High (≥10)</td>
<td>10,855</td>
<td>10,855</td>
<td>2,535,138</td>
</tr>
<tr>
<td>Med (1-10)</td>
<td>8,268</td>
<td>8,268</td>
<td>12,805</td>
</tr>
<tr>
<td>Very Low (&lt;0.2)</td>
<td>1,193</td>
<td>1,193</td>
<td>7,325</td>
</tr>
</tbody>
</table>

Red and orange lettering indicates likely false-positives, i.e. annotated introns never observed and/or excised in any sample in this study; green lettering indicates false negatives, i.e. unannotated introns observed at levels consistent with transcript abundance. A, Intron abundance in tachyzoites, gametocytes, or any sample, stratified by splicing efficiency and annotation status. Green, red and blue shading reflects the relative abundance of annotated, unannotated, and non-genic introns in each sample. B, Stratification of all introns (from right-most column in panel A) based on the abundance, splicing efficiency and annotation status of alternative (overlapping)

36
introns. *Orange* shading reflects unannotated introns that should probably be considered alternative splice junctions; *green*, currently unannotated introns that should replace the existing annotation; *red*, annotated introns that should be removed from the current annotation; *blue*, annotated introns that should be downgraded due to more abundant unannotated alternatives.

All of the HXGPRT introns discussed above (*cf.* Fig 2A) are appropriately represented in Fig 2.4 (*magenta* circles; filled = annotated; open = unannotated). The three introns that are always excised (I, IV & V) lie close to the ISRPM = FPKM diagonal, while introns II & III, which define the exon cassette responsible for HXGPRT isoform II, are less abundant (ISRPM/FPKM ~0.3), as is intron II-III, representing the alternative exon-skip variant responsible for HXGPRT isoform I (open circle). All three of these alternatively-spliced introns still lie within the true-positive sector (ISRPM >1 and >20% FPKM), but less plausible HXGPRT introns do not (including several that are off-scale on this plot). While most annotated introns lie within the true-positive sector in this CZ-H3 tachyzoite sample, 8133 appear as possible false positives in Fig 2.4, *i.e.* ISRPM <1 and/or ISRPM/FPKM <0.2. Considering other samples, however, reveals that the majority of these introns are expressed in other life cycle stages, as discussed below.

For example, while a similar distribution of annotated and unannotated introns is observed in gametocyte stage parasites (Fig 2.5A, Table 2A), most of the apparent tachyzoite stage false-positives are abundantly expressed and efficiently excised in gametocytes (*orange* datapoints in Fig 2.5). ~79% of all annotated introns are supported in tachyzoites, and 85% in gametocytes, but only 73% were observed in both life cycle stages, while 6-12% were stage-specific (Table 2A). Combining all 20 of the high quality datasets used in this study (green shading in Table 1) and plotting the maximum evidence for each intron (in any experiment) against transcript abundance in that experi-
ment (Fig 2.5B), provides strong support for 93% of all annotated introns at ISRPM ≥1 and ISRPM/FPKM ≥0.2, and 75% at ISRPM ≥10 (Table 2A), i.e. there are relatively few false positives in the current T. gondii reference annotation. It is likely that more extensive sequencing, including additional strains, life cycle stages, conditions and treatments would reduce this number still further.

**Figure 2.5.** Genome-wide identification of implausible annotated introns (FP).

Intron-spanning read abundance (ISRPM) as a function of transcript coverage (FPKM) for annotated introns (black) and novel (unannotated) introns (gray). A, T. gondii gametocytes display a similar pattern with most introns observed at levels comparable to transcript abundance being annotated, i.e. black dots lie along the diagonal, but many introns not expressed in tachyzoites (black dots at bottom or below the diagonal in Fig 2.4) are expressed in gametocytes (orange), often at transcriptionally-appropriate levels. B, ISRPM vs FPKM values for all putative introns, in whichever sample that intron is maximally evident; note that this provides further separation of black and gray dots, leaving few false positives.

Potential false negatives, i.e. unannotated introns that map to genes with a maximum abundance consistent with mRNA levels (~7% of plausible intron candidates), are represented by gray datapoints overlapping the dominant black cloud in Fig 2.5B. It is
interesting that most of these candidate splice junctions display somewhat lower abundance and/or inefficient excision relative to expectations based on FPKM values, \textit{i.e.} they are slightly offset towards lower edge of the black diagonal cloud representing annotated introns (true positives). This observation suggests that they may represent subdominant splice variants rather than fully false negatives, as is the case for introns attributable to HXGPRT exon-skip variants. (Note that HXGPRT is very highly expressed in gametocytes, at levels \textasciitilde20-fold greater than in tachyzoites; compare pink dots in Fig 2.5 \\& 2.4.)

To directly assess the relationship between possible false negatives and existing intron annotation, we compared the maximal excision efficiency (ISRPM/FPKM) for each intron (vertical axis in Fig 2.6) with the excision efficiency of the maximum \textit{alternative} overlapping intron (horizontal axis). The four quadrants of this figure, defined by excision efficiencies of greater or less than 20\% in the reference vs overlapping alternative introns represent:

- \textit{Top Left}: Introns expressed and excised at comparable levels (ISRPM/FPKM \textasciimath{\approx} 1) and overlapping alternative introns that are \textasciimath{\sim}5-5000-fold less efficiently spliced. This quadrant is dominated by annotated introns (\textit{black}), including the efficiently-spliced HXGPRT introns I, IV \& V. Many additional annotated introns lack any plausible overlapping alternative (off-scale to the left).

- \textit{Bottom Right}: Introns that are inefficiently spliced but overlap alternatives excised at expected levels, \textit{i.e.} the reciprocal of the above. This quadrant is dominated by unannotated introns (\textit{blue}), including implausible alternatives to HXGPRT introns I, IV \& V (\textit{cf.} Figs 1A\&C), and many more off-scale low.

- \textit{Bottom Left (gray shading)}: Low abundance and/or inefficiently spliced introns that overlap alternatives that are also low abundance and/or inefficiently spliced (\textit{gray}).
• **Top Right (yellow shading):** Introns that are abundant and efficiently spliced, and overlap alternatives that are also abundant and efficiently spliced, such as those for HXGPRT introns II & III (*solid magenta dots*) and the exon-skip variant represented by HXGPRT intron II-III (*open magenta circle*). (Note that stage-specific alternative exons would be expected to be even more efficiently excised.)

Figure 2.6. Genome-wide identification of likely unannotated introns (FN), including alternatively-spliced isoforms.

Plotting splicing efficiency (ISRPM/FPKM) from Fig 2.5B against splicing efficiency for the most prevalent overlapping alternative (Additional File 1) identifies unannotated introns that should be considered for inclusion in the reference annotation (~7.2% of annotated introns), including likely splice variants (~3.6% of annotated introns; *yellow quadrant*); *Black dots* indicate annotated introns overlapping unannotated alternatives; *blue dots* indicate unannotated introns overlapping
annotated alternatives, and gray dots denote unannotated introns overlapping unannotated alternatives. See text for further discussion.

Summary statistics for overlapping introns are provided in Table 2B. Considering Tables 2A & 2B in aggregate, and comparing with Fig 2.6, permits an objective analysis of current annotation status, using metrics that should also be applicable to other systems:

- **True Positives:** 93% of *T. gondii* introns annotated in ToxoDB release 28 are abundantly or moderately expressed and efficiently excised in at least one experimental sample (black datapoints in the upper half of Fig 2.6): 29,584 display maximal abundance ≥10 ISRPM and splicing efficiency (ISRPM/FPKM) ≥20%; 7325 display maximal ISRPM from 1-10 (and ISRPM/FPKM ≥20%). 84% of these (23,983 + 6957) show no overlap with any plausible alternative intron (off-scale left in Fig 2.6). 5969 overlap plausible alternatives, but 78% of those alternatives (4569 + 72) are only weakly expressed and/or inefficiently excised (horizontal black cloud in Fig 2.6). Just 1328 plausibly-expressed and efficiently-spliced annotated introns were found to overlap plausible unannotated alternatives (black dots within the yellow shaded region). Among these, the annotated intron was most prominent 75% of the time (838 + 164; orange shading in Table 2B; above the diagonal in Fig 2.6). Only 326 (194 + 132) overlap unannotated introns that are more prominent than well-supported annotated introns (green shading in Table 2B; black dots below the diagonal in the yellow-shaded region of Fig 2.6).

- **False Positives:** 2744 annotated introns (red & orange lettering in Table 2A) were identified as likely false positives due to low evidence (1677) and/or inefficient excision (1208), and are represented by black dots in the lower half of Fig 2.6. 576 overlap plausible unannotated introns that could be considered as replacement annotation (red/orange letting and green shading in Table 2B; black dots in the lower right-hand quadrant of Fig 2.6). Many of these share either a donor or acceptor splice site with the unsupported annotation (see below).
• **False Negatives:** 6922 unannotated introns map to genes in the current reference annotation and are abundantly expressed in at least one sample, but only 41% of these (1108 + 1739) are also efficiently excised. Most (1444; 51%) overlap plausible alternative introns (gray & blue dots in the upper half of Fig 2.6), which are usually more probable annotated introns (681 + 285 = 966; blue dots below the diagonal in the yellow shaded region of Fig 2.6). A few (151 + 123 = 274) represent introns that are more probable alternatives to the existing annotation (blue dots above the diagonal in the yellow shaded region in Fig 2.6; blue shading in Table 2B), and fewer still should probably supplant the existing annotation (just 114 + 50 = 164; blue dots in the upper-left quadrant, the reciprocal of black dots in the lower right-hand quadrant, described above; red shading in Table 2B). In addition, 302 plausible unannotated introns overlap unannotated alternatives (gray dots in the upper half of Fig 2.6), and 1141 high confidence unannotated introns do not overlap other plausible introns (Table 2B; off-scale left in Fig 2.6).

• **True Negatives:** Even excluding the millions of very low abundance junctions previously discarded, most unannotated introns are not significantly expressed and/or processed, in any sample (black numbers in the right-hand column of Table 2A).

• **Intergenic Introns:** Introns observed in RNA-seq experiments that fail to map to annotated genes are relatively rare, but 1230 + 3809 = 5039 display plausible abundance (Table 2B), suggesting extended gene models or previously unannotated genes. Many of these are most evident in non-tachyzoite stage parasites, suggesting stage-specific expression, and reflecting the dominance of data from the acutely-lytic *in vitro* tachyzoites in generating the current reference annotation.

**Implications for *T. gondii* annotation**

Exploiting the parameters defined above, various transformations and filtering of data in Additional File 1 were used to identify potential cases of erroneous annotation in the reference *T. gondii* genome, including alternative splice variants and novel gene models. Representative examples are provided in Figs 2.7-11 (and highlighted in Figs
These results are currently being incorporated into the reference genome database.

*TgME49_234450* (Fig 2.7) is annotated as ribosomal small subunit protein Rps15A, based on highly conserved protein sequence motifs across all species. The current structural annotation describes two introns, but overlapping alternatives (defined based on the analysis presented in Fig 2.6) reveal a skipped exon within the first intron (*magenta star*) in ~30% of tachyzoite transcripts. This organization (and abundance) is similar to the HXGPRT cassette exon (Fig 2.1), except that the reference annotation for HXGPRT includes the cassette exon, whereas the annotated RPS15A transcript does not. In contrast to the 147 nt HXGPRT cassette, which encodes 49 amino acids, the 69 nt RPS15 cassette exon is predicted to result in premature protein translation, due to an internal stop codon (Supplementary Fig S2). Close inspection of the genome sequence and multiple sequence alignments reveals that the annotated translation initiation site is likely incorrect. An alternative ATG immediately downstream of the cassette exon offers better sequence context for translation and protein stability (Matrajt et al. 2004), and yields a product that aligns with RPS15A proteins from other species across its entire 130 amino acid length (Fig 2.7, bottom).
Figure 2.7. Genome browser view & multiple sequence alignment of TgRps15a.

Top, Genome annotation and selected RNA-seq tracks from tachyzoites, bradyzoites, and/or gametocytes (as labeled). Note that solid blue tracks are scaled logarithmically while tracks
shown in semi-transparent overlay are linear (also note different scales, selected to highlight alternative splicing). As in Fig 2.1, arrows and circles indicate transcript termini, stars indicate variants discussed in the text. In contrast to Fig 2.1, two tracks display candidate introns (brackets): the ‘filtered’ track is restricted to display only those introns that pass the abundance and efficiency criteria described in this dissertation. **Bottom,** Multiple sequence alignment of *TgRPS15a* with various other species. Current annotation includes a 101 amino acid N-terminal exon (*yellow*) upstream of the most plausible initiation codon (*green*); inclusion of the alternatively-spliced cassette exon noted above would introduce 10 amino acids ending in a premature termination codon (*turquoise*).

Moreover, while proteomics evidence provides ample support for the body of the protein (Wastling et al. 2009; and other data in ToxoDB.org), there is no evidence for translation of the 101 amino acid N-terminal extension predicted by the current annotation. In sum, there is strong support for an exon-skip variant at this locus, and inclusion of the cassette may be stage-specific (more common in bradyzoites, less common in gametocytes), but these alternative splice products likely affect the 5′UTR only. As indicated by the RNA-seq data, and supported by the presence of chromatin activation marks (Gissot et al. 2007; ToxoDB.org) the annotated transcription start site corresponds to the longest potential transcript, but not the most abundant. The annotated UTR represents <0.1% of transcripts; most transcription initiates >500 nt downstream, just upstream of intron I.

The availability of RNA-seq data dramatically improves *T. gondii* UTR annotation (cf. Fig 2.2), but in general, the algorithms employed (Bernal, Crammer, and Pereira 2012; Bernal and Pereira 2012) appear to be too greedy, annotating the longest, rather than the most abundant UTRs. Transcript initiation sites, including the use of stage-specific promoters, can dramatically impact gene models, as it is of course impossible to excise sequences that are not transcribed. Transforming the reference data (Additional
File 1) to examine stage-specific intron usage (see below, Fig 2.13) highlights Isocitrate Dehydrogenase (TgME49_266760), among many other genes. As shown in Fig 2.8, this gene appears to be transcribed from two promoters: a low-level constitutive promoter, and a 20-fold more potent gametocyte-specific promoter ~800 nt downstream, within intron I.

Figure 2.8. Genome browser view of alternatively-spliced Isocitrate DH.
Genome annotation and selected RNA-seq tracks from tachyzoites and gametocytes. Note that red tracks are scaled logarithmically, while tracks shown in semi-transparent overlay are linear (also note different scales, selected to highlight alternative splicing). Arrows and circles indicate transcript termini, stars indicate variants discussed in the text. Two tracks display candidate introns (brackets): the 'filtered' track is restricted to display only those introns that pass the abundance and efficiency criteria described in this dissertation.

As a result, tachyzoite transcripts match the annotated gene model, but gametocyte transcripts lack exon I, initiating within the intron and therefore containing a longer "second" exon. As observed for RPS15A (Fig 2.7), multiple sequence alignment (Supplementary Fig S3) and analysis of available proteomics (Xia et al. 2008) data suggests that the annotated translation initiation codon for TgME49_266760 is incorrect, and
translation begins within the second exon, downstream of intron I, yielding the same mature protein product in both tachyzoite and gametocyte-stage parasites.

Although relatively infrequent, alternative splicing that affects the protein coding sequence is occasionally observed, as in TgME49_278830, which encodes glucose-6-phosphate dihydrogenase (Fig 2.9A, displaying just the first 4 exons of this 21 exon transcript). Multiple promoters yield a diversity of 5’ ends, affecting the selection of splice donors for intron I (which lies entirely within the UTR). Two alternatives were also observed for Intron II, which lies within the protein coding sequence: the annotated intron predominates, but a splice acceptor variant is also observed, resulting in 60 nt of additional coding sequence adding 20 amino acids to the mature protein (magenta star) (Supplementary Fig S4). In contrast to the examples cited above, the annotated translation start displays plausible sequence context (although an in-frame ATG 48 nt downstream within the same exon is even better), and no alternative initiation codon is present upstream of highly conserved protein sequences. Experimental proteomics evidence also confirms supports usage of the second ATG (Xia et al. 2008).

As indicated in Fig 2.9B, the alternative (longer) G6PDH transcript was observed in tachyzoites from multiple parasite strains, but not in mature bradyzoites, gametocytes, or unsporulated or sporulated oocysts (also seen in bradyzoite samples generated in vitro, which contain numerous tachyzoites; Dzierszinski et al. 2004; Soete, Camus, and Dubremetz 1993). Interestingly, in two separate time courses examining transcription at various times post-infection, utilization of the alternative splice acceptor was most common early during intracellular replication (see Supplementary Fig S5 data for all
RNA-seq data on this differentially-spliced intron, including multiple strains with lower coverage data).

**Figure 2.9. Genome browser view of alternatively-spliced G6PDH and Intron II abundance.**

A. Genome annotation and selected RNA-seq tracks from tachyzoites and gametocytes. Note that blue tracks are scaled logarithmically, while tracks shown in semi-transparent overlay are linear (note different scales, selected to highlight alternative splicing). *Arrows and circles* indicate transcript termini, *stars* indicate variants discussed in the text. Two tracks display candidate introns (brackets): the ‘filtered’ track is restricted to display only those introns passing the abundance and efficiency criteria described in this thesis. B. G6PDH Intron II abundance. Intron excision frequency for G6PDH splice acceptor variants for all RNA-seq samples (Table 1), stratified by parasite strain, time post-infection (for tachyzoites), and life cycle stage.

As noted in Table 2, several thousand introns map to the *T. gondii* genome outside of annotated genes, and display abundance consistent with splice sites for known genes. In order to estimate the number of unannotated protein-coding genes in the parasite genome, we examined a randomly selected subset of all intergenic introns, and also a subset of those with ISRPM >35. In addition, we manually curated all intergenic regions within a 1 Mb chromosomal span. All of these analyses suggest that the current reference genome annotation is lacking ~300 protein-coding genes (including single exon genes), most of which are specifically expressed in non-tachyzoite stages, for
which data has only recently become available (from this study and others). These unannotated genes account for ~1/3 of all plausible intergenic introns. For example, Fig 2.10 shows a 5-exon gene on chromosome Ia that is expressed in gametocytes only (green shading). An additional third of intergenic introns can be attributed to unannotated exons extending existing gene models (often as UTR exons), and most of the remaining third are associated with non-coding RNAs of unknown significance (and often inefficiently excised).

![TgME49_chrIa:87,001..93,000 Unannotated Gene](image)

**Figure 2.10. Genome browser view of an unannotated gene.**

Genome annotation and selected RNA-seq tracks from tachyzoites and gametocytes. Note that blue and red tracks are scaled logarithmically. Arrows and circles indicate transcript termini. Two tracks display candidate introns (brackets): the ‘filtered’ track is restricted to display only those introns that pass the abundance and efficiency criteria described in this thesis.

Overall, the most common errors associated with existing annotation derive from inaccurate annotation of transcript termini, which are often stage-specific. As noted above, this may impact intron usage, splicing efficiency, and the selection of translation
start sites. In the absence of functional evidence, however, it is not necessarily clear that alternative starts and stops are biologically relevant, or should be annotated. For example, the dynein heavy-chain family protein TgME49_309980 (Fig 2.11A) is a 63 exon gene, spanning nearly 50 kb. Despite low expression levels (FPKM <10), all 62 introns appear to be accurately annotated, but tachyzoite stage-specific termini suggest a much shorter transcript in this stage, including exons XXXVII-LIII only (or perhaps XIX-LXV), and lacking many domains likely to be essential for function. From the available data (all based on steady-state RNA, as ribosomal profiling data is not yet available for T. gondii), it is unclear whether the different transcript termini inferred from tachyzoite vs gametocyte RNA-seq data reflect differences in transcriptional initiation and termination, or differences in mRNA stability and degradation, but chromatin marks provide some evidence in support of a shorter tachyzoite-specific transcript (not shown). In addition, a tachyzoite-specific antisense RNA transcript (IncRNA; green box in Fig. 2.11) could potentially play a role in regulating mRNA stability (discussed further below).

While most genes in the T. gondii genome are accurately annotated, and most high abundance unannotated introns are readily interpretable (as noted above), some genes will remain refractory to annotation without additional experimental data. For example, TgME49_270520 (Fig 2.11B) encodes a ‘hypothetical protein’ of no known function that is most abundant in gametocytes but expressed in all life cycle stages except unsporulated oocysts. The current reference annotation includes 6 exons, but at least 2 additional exons are evident (green stars); analysis of intron-spanning reads suggests that exons IV-VII may be included in the mature mRNA in various permutations and combinations, possibly using different combinatorial patterns in different life cycle stages.
Figure 2.11. Genome browser view of alternatively-spliced transcripts that are difficult to confidently annotate.

Genome annotation and selected RNA-seq tracks from tachyzoites and gametocytes. Note that blue and red tracks are scaled logarithmically, while tracks shown in semi-transparent overlay are linear (also note different scales, selected to highlight alternative splicing). Arrows & circles indicate transcript termini, stars indicate variants discussed in the text. Two tracks display candidate introns (brackets): the ‘filtered’ track is restricted to display only those introns that pass the abundance and efficiency criteria described in this dissertation.

Figure 2.12 reproduces Fig 2.6, highlighting the expression of annotated and unannotated introns for some of the alternatively-spliced genes discussed above. Note that most annotated introns (closed circles) only overlap alternatives at implausibly low
abundance, i.e. they map to the upper left quadrant, while most unannotated introns (open circles) map to the lower right quadrant, i.e. they overlap annotated introns that are much more efficiently expressed and spliced.

Figure 2.12. Identification of alternative-spliced introns.
Maximum splicing efficiency (ISRP/M/FPKM) for each putative intron vs its most prominent overlapping alternative intron (replica of Fig 2.6). Black, annotated introns overlapping unannotated alternatives; Blue-gray, unannotated introns overlapping annotated alternatives; Gray, unannotated introns overlapping unannotated alternatives. Colored dots highlight introns associated with TgME49_200320 (HXGPRT; Magenta … see Fig 2.1), TgME49_234450 (Rps15a; Red … see Fig 2.7), TgME49_278830 (G6PDH; Turquoise … see Fig 2.9), TgME49_270520 (hypothetical protein; Orange … see Fig 2.11B).
Alternatively-spliced introns map to the yellow shaded area, including the two annotated HXGPRT introns and the single unannotated cassette exon-spanning intron (magenta), the single annotated exon-spanning intron in Rps15A and the two unannotated introns flanking the cassette exon (red), and both the annotated and unannotated splice acceptor variants for G6PDH (turquoise). Additional annotated HXGPRT and G6PDH introns are efficiently spliced, but lack any alternatives, and are therefore off-scale to the left. Several unannotated G6PDH introns overlap the first annotated intron, within the 5’UTR (see Fig 2.9), and map to the lower right quadrant. The first two TgME49_270520 introns are excised in all splice variants (see Fig 2.11B) and are represented by solid orange dots in the upper left quadrant, but other annotated and many unannotated introns combining cassette exons in various ways yield a diversity of introns displaying low apparent splicing efficiency.

Figure 2.13 highlights stage-specific alternatively spliced introns, by comparing the intron excision efficiency (ISRPM/FPKM) of tachyzoites vs gametocytes. For example, Isocitrate DH intron I (TgME49_266760; left-most green dot) is inefficiently excised in gametocytes only because the gametocyte stage-specific promoter lies within the first intron (see Fig 2.8). Many such intron isoforms map to UTRs, often incorrectly annotated (as in this case). Because only the central portion of TgME49_309980 (Dynein HC; see Fig 2.11A) is transcribed in tachyzoites, gene-level FPKM values are low, central introns appear to be excised with efficiencies >1, and introns outside this region appear to be excised unusually poorly (blue dots in Fig 2.13). The unannotated intron acceptor variant in G6PDH (turquoise; see Fig 2.9A) is not observed in gametocytes (Figure 2.9B), and therefore lies on the vertical axis in Fig 2.13.
Figure 2.13. Identification of stage-specific alternative-spliced introns.

The size of the dots in this panel reflects the maximal ISRPM observed in these samples. Yellow highlights introns displaying FPKM values >1 in both samples, maximum intron abundance (in either sample) >1, and >5-fold differential intron excision efficiency between tachyzoites and gametocytes. Introns labelled in magenta, red, turquoise and orange show no significant stage-specificity in intron excision, except as noted in the text.

Discussion

A previous report has demonstrated the value of RNA-seq data for improving T. gondii genome annotation, including the recognition of alternatively-spliced isoforms (Hassan et al. 2012). Because our preliminary analysis revealed that strand-specific
RNA-seq data is substantially more valuable for gene model assessment (cf. Fig 2.3), detailed studies of structural annotation and alternative splicing were carried out using the 20 highest quality, strand-specific datasets (total of >500M reads, including >60M intron-spanning reads; Table 1). Results were then integrated with all available information, including non-strand-specific and/or lower depth RNA-seq data (Lorenzi et al. 2016; Minot et al. 2012; Reid et al. 2012; Pittman, Aliota, and Knoll 2014; Hehl et al. 2015), as well as microarray (Fritz et al. 2012; Grigg et al. 2001; Behnke et al. 2014; Buchholz et al. 2011; Bahl et al. 2010), SAGE tag & EST studies (Radke et al. 2005), proteomics data (Xia et al. 2008), chromatin marks (Gissot et al. 2007), population-level sequence variation (SNPs; Lorenzi et al. 2016), etc. These results were consistent with all previous reports on transcript variation, including alternative splicing of the HXGPRT locus (Fig 2.1; Donald et al. 1996; Chaudhary et al. 2005), and examples highlighted by Hassan et al. 2012.

Overall, the accuracy of existing reference annotation is quite high: >93% of all annotated introns are supported by the pooled mRNA-seq datasets. In aggregate, these samples define >2.7M intron junctions, but while almost all correspond to consensus intron boundaries and are therefore likely to be genuine, most are present at very low abundance (<<0.1 intron-spanning reads per million; ISRPM). Establishing a minimum requirement of ISRPM >1 appears to be quite conservative, removing very few annotated introns, but excluding the vast majority of unannotated introns (Figs 2.4-2.6, Additional File 1). In addition, it is also helpful to exclude introns that are inefficiently excised (low ISRPM/FPKM ratios; also known as $\psi$ (Percent Spliced In; Venables et al. 2008), even if they are frequently observed in heavily transcribed genes. Various statistical methods have been developed to improve the estimation of $\psi$ (Katz et al.
ISRPM/FPKM serves as an adequate proxy when gene annotation is close to accurate, and is easier to automatically extract from RNA-seq mapping pipelines and genome databases. Assuming that the current *T. gondii* annotation provides a reasonable approximation of the truth, with little systematic bias, setting the minimum excision efficiency at 20% minimizes false discovery (Fig 2.6, Fig S1) … although of course some less frequent isoforms may still be functional, or may be observed at higher frequency under as-yet-untested conditions.

Plotting intron frequency vs transcript abundance clearly distinguishes introns that are always excised from those that are alternatively spliced, and those with low penetrance that are unlikely to yield biologically-functional transcripts (Fig 2.4; see HXGPRT as a positive control; Uhlén et al. 2015). Most of the putative false positives in this example are in fact expressed in other life cycle stages (Table 2, Fig 2.5A). Defining each intron based on the sample providing maximum support permits identification of >93% of all annotated introns (Table 2, Fig 2.5B). A modest number of annotated introns (2693) have no support and should probably be removed from the official annotation, and a similar number (2847) should be added, either as novel introns (1239), or in conjunction with other unannotated introns (204), secondarily to an annotated intron (966), as the preferred isoform (274), or in a few cases replacing an annotated intron (164). Plotting ISRPM/FPKM ratios in reference introns vs the most prominent alternative isoform (if one exists) provides a more sensitive means for discriminating alternatively-spliced introns (Figs 2.6, 2.12 & 2.13). These criteria are readily automated, permitting application to many incompletely annotated genomes. For example, the filtering characteristics based on those described above have recently been imple-
mented in ToxoDB build 29 (compare the two intron tracks presented in Figs 2.7-2.11 with minimally-filtered splice junction data in Figs 2.1 & 2.3), and appear applicable to *Plasmodium falciparum* as well (see Chapter 4).

Case studies reveal the same assortment of incomplete annotations observed in other eukaryotes (Mudge and Harrow 2016; Hassan et al. 2012), including UTR information, missing exons (especially at transcript termini), and the usual assortment of splice variants: alternative splice donors, splice acceptors, exon-skip variants, unexcised introns, etc (Figs 2.7-2.11). A plethora of overlapping but inefficiently spliced introns suggests multiple promoters, frequently associated with a UTR (cf. Figs 2.7, 2.8 & 2.9), and often resulting in mis-annotation of the most probable translational start (ATG).

Indeed, it seems likely that improper ATG annotation constitutes the most biologically-relevant source of errors in current *T. gondii* annotation, as accurate definition of the mature N-terminus has important implications for subcellular localization predictions, and trafficking is particularly important for mediating pathogen interactions with their host cells. The sequence context of *T. gondii* initiation codons is known to be highly constrained (Matrajt et al. 2004), providing a basis for systematic improvement of CDS annotation. UTR predictions could also be improved substantially by using a less greedy algorithm to select the most abundant UTR(s), rather than the longest (cf. Fig 2.7). Similarly, gene annotations that incorporate the most abundant introns, rather than the one that results in the longest open reading frame, as is currently the case, would yield more accurate gene models. Additional evidence based on chromatin marks (Gissot et al. 2007) and proteomics studies (Xia et al. 2008) can provide further support for (or refutation of) candidate gene models, but ultimately, there is no substitute for biologists understanding and being able to interpret the underlying data. Without additional
experimental evidence, it is unlikely that any algorithm will be able to accurately annotate biologically significant transcriptional start sites for TgME49\_288830 (Fig. 2.9), or sensible structural models for TgME49\_270520 (Fig. 2.11B),

In the absence of gene assignments (and therefore FPKM values), evaluation of introns that map outside annotated genes is more difficult, but this problem could be addressed by using local analyses (Katz et al. 2010; Shen et al. 2014; Trapnell et al. 2013; Vaquero-Garcia et al. 2016). On the order of 300 protein-coding genes appear to be missing from the current annotation, but now that data is available for most life cycle stages, it is likely that many could be identified by re-running de novo gene predictors, informed by newly available RNA-seq datasets. This would be especially true since current de novo gene finders have used evidence exclusively from the tachyzoite stage and genes expressed in other non-tachyzoite stages have been predicted based on ab initio gene finders only. Other transcripts evident within intergenic regions (both with and without introns) may highlight long non-coding RNAs. Defining these IncRNAs has proved difficult however, as gene finders cannot rely on the same sequence features known to be useful for protein coding genes.
CHAPTER 3: MECHANISMS OF TRANSCRIPTIONAL REGULATION IN 
TOXOPLASMA GONDII (Adapted From Paper)

The development and dissemination of low-cost technologies for deep sequencing of RNAs has yielded many new insights (Wang, Gerstein, and Snyder 2009). mRNA-seq provides experimental evidence for genome annotation, enabling identification of differentially-spliced transcript isoforms, and previously-unrecognized coding and non-coding RNAs, as described in Chapter 2 (Mudge and Harrow 2016), while also providing a comprehensive, quantitative picture of transcript diversity. Whole transcriptome profiling of various cells and tissues, under diverse conditions, permits the identification of tissue / lineage / stage / strain-specific signatures (Uhlén et al. 2015), which can be used to construct metabolic and physiological models (de Oliveira Dal'Molin et al. 2016), examine complex samples such as pathogen-infected tissues (Westermann, Gorski, and Vogel 2012), etc.

Although comparing global gene expression patterns was not the primary goal of this study, we have generated and sequenced 24 strand-specific RNA libraries from several developmental stages/strains, as noted above, and examined these in conjunction with 45 additional libraries (Table 1). In aggregate, this provides the most comprehensive collection of data available on T. gondii transcript abundance across the parasite life cycle. Overall, transcriptomic results are highly consistent with biological and phenotypic variation across the complex parasite life cycle (Fig 1), including previously undescribed differences in gene expression during intracellular tachyzoite replication. Strong circumstantial evidence also suggests that lncRNAs may play an important role in regulating stage-specific expression during sexual differentiation and sporogony.
Methods

Data analysis and visualization

FPKM values for all genes (8,920 rows in Additional File 3) in the *T. gondii* genome (ToxoDB version 28) were filtered to restrict to those with FPKM value ≥ 20 (5,645 genes) for PCA and MDS analysis using the statistical excel add-in XLSTAT (Addinsoft; Figs 3.1, 3.2). Pearson (n) standardization was used for PCA, between-sample proximity matrices were calculated from the factor scores based on Euclidean distances. For MDS, we used the proximity matrix from PCA factor scores and selected the absolute model, Kruskal's stress, random initial configuration, 10 repetitions and stopped conditions if convergence was equal to $10^{-5}$ with > 500 iterations.

Results

Stage-specificity of *T. gondii* gene expression

RNA-seq datasets in this study were exploited to examine how global gene expression patterns compare across the parasite life cycle, from the acutely lytic tachyzoites responsible for disease, to latent bradyzoite tissue cysts in the brain, to sexual stage gametocytes from feline intestinal epithelium, to unsporulated oocysts in the environment (the fertilized zygote), and finally to the sporulated meiotic progeny (sporozoites) that are the immediate precursors to reinitiation of tachyzoite infection (Dubey, Lindsay, and Speer 1998). Additional File 3 presents FPKM values for the entire *T. gondii* genome for the 20 high quality strand-specific RNA-seq experiments highlighted in Table 1. Sample-specific correlations were performed using all genes that are expressed at FPKM >20, in any sample (63% of the genome).
Principal component analysis (PCA) clearly separates major life cycle stages, correlating well with parasite biology (Table 3). PC1 accounts for 27% of variation, and readily distinguishes oocysts (both sporulated & unsporulated) from gametocytes from asexual stages (both tachyzoites & bradyzoites). PC2 (23%) distinguishes gametocytes and unsporulated oocysts from tachyzoites/bradyzoites & sporozoites (see Fig 3.1 for a plot of PC1 vs PC2). PC3 (12%) distinguishes sporulated vs unsporulated oocysts; PC4 (11%) appears to distinguish early vs late stage tachyzoites. PC5 (8%) distinguishes mature bradyzoites from other stages. Overall, the top 10 principal components explain ~97% of observed variation between the 20 samples included in this study.

### Table 3. Analysis of stage-specific gene expression patterns in *T. gondii* by Principal Component Analysis.

Principal Component Analysis (PCA) for the 20 high quality strand-specific RNA-seq samples defined in Table 1, using all genes that are ever expressed at FPKM >20, in any sample. Contributions of each sample to the first 10 principal components, accounting for ~97% of observed variation.

<table>
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<th>Sample</th>
<th>PC 1</th>
<th>PC 2</th>
<th>PC 3</th>
<th>PC 4</th>
<th>PC 5</th>
<th>PC 6</th>
<th>PC 7</th>
<th>PC 8</th>
<th>PC 9</th>
<th>PC 10</th>
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<td></td>
<td>26.7%</td>
<td>23.1%</td>
<td>11.7%</td>
<td>11.4%</td>
<td>7.9%</td>
<td>5.2%</td>
<td>3.2%</td>
<td>2.5%</td>
<td>2.2%</td>
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<td>-2.53</td>
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<td>9.17</td>
<td>-11.19</td>
<td>-2.37</td>
<td>11.83</td>
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<td>-10.83</td>
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<td>-14.32</td>
<td>-0.59</td>
<td>-4.53</td>
<td>22.11</td>
</tr>
<tr>
<td>2Tz44_S</td>
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<td>-39.66</td>
<td>-6.37</td>
<td>28.90</td>
<td>-7.18</td>
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<td>-16.98</td>
<td>5.77</td>
<td>10.07</td>
</tr>
<tr>
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<td>-6.33</td>
<td>-19.12</td>
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<td>6.65</td>
<td>7.10</td>
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<td>9.16</td>
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<td>-3.93</td>
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<td>18.85</td>
<td>-3.15</td>
<td>12.15</td>
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Figure 3.1. Spatial distributions of stage-specific gene expression patterns by PCA analysis. First two principal components for all samples, colored to highlight tachyzoites (green), bradyzoites (orange), gametocytes (red), unsporulated oocysts (magenta) & sporulated oocysts (purple).

See Additional File 3 for further details including eigenvalues, sample correlations, lists of specific genes making the greatest contribution to these principal components, eigenvectors for all genes, and the results of other analyses (K-means clustering, agglomerative hierarchical clustering, hierarchically-clustered expression heat maps, etc).

Little is known about the specific genes or mechanisms involved in sexual differentiation in *Toxoplasma*, so it is difficult to compare these results with prior knowledge, but bradyzoite differentiation has been studied extensively (Dzierszinski et al. 2004; Soete, Camus, and Dubremetz 1993; Singh, Brewer, and Boothroyd 2002), and it is reassuring to find that major contributors to PC5, which clearly distinguishes mature bradyzoites from other stages, include the known bradyzoite differentiation markers BAG1, LDH2, ENO1, SRS9, SRS35A & SRS35B (Wasmuth et al. 2012; Dzierszinski et al. 2001).
Fig 3.2 uses multidimensional scaling (MDS; Hout, Papesh, and Goldinger 2013) to more accurately reflect between-sample distances in two dimensions (although the axes themselves are meaningless), particularly for various strains and stages of tachyzoites, which are not well-distinguished by PC1 & 2. Note that all 12 tachyzoite samples group together (green cloud), despite representing three strains, including the canonical type II & III lineages (green & blue dots, respectively; Howe and Sibley 1995; Grigg et al. 2001), and various early to late time points during intracellular replication. Additional analysis (not shown) demonstrates that type I strain tachyzoites co-cluster as well. *In vitro* bradyzoites (2Bz_S) cluster with tachyzoites (although in the direction of mature bradyzoites isolated from murine brain cysts; 3Bz_S, *orange*), as expected given the long, slow process of tachyzoite-to-bradyzoite differentiation (Dzierszinski et al. 2004; Soete, Camus, and Dubremetz 1993).

**Figure 3.2. Spatial distributions of stage-specific gene expression patterns by MDS analysis.**

MDS of the same samples; that for tachyzoites, samples group based on time post-infection (hr 2, 4, 8 …) rather than parasite strain (2Tz=ME49, 3Tz=VEG).

Gametocytes (*red*), unsporulated oocysts (*magenta*), and sporulated oocysts (*purple*) are also readily distinguished, justifying the binning of these samples, as shown.
in Fig 3.3 (green and blue dots represent tachyzoites samples binned by strain; black dot represents pooled data for all *T. gondii* tachyzoite samples).

Figure 3.3. Spatial distributions of stage-specific gene expression patterns by MDS analysis binned by stage and strains.
MDS for samples binned samples by stage and strain.

It is interesting to note that tachyzoite samples from similar time points post-infection are often more similar to each other than early vs late time points from the same strains in Fig 3.2, *i.e.* the 8 hr post-infection samples of ME49 vs VEG parasites (2Tz8 & 3Tz8) are relatively similar to each other, as are 36 & 44 hr time points. In contrast, ME49 strain 8 & 36 hr time points are quite distinct, as are VEG strain 8 & 36 hr time points. PCA analysis of samples binned based on time post-infection (Fig 3.4) shows that PC1 & 2 account for >76% of the observed variance.

Principal component 1 (PC1) does a good job of stratifying samples based on early vs late infection, and the top indicators are slightly enriched in genes associated with intracellular trafficking (*P*<10^{-3}); PC2 correlates with intracellular vs extracellular tachyzoites, and is enriched in genes bearing GO terms associated with redox balance and...
chromatin assembly ($P<10^{-3}$). Functional distinctions over the course of intracellular
tachyzoite infection have not been extensively studied, but analysis of genomic-scale
expression profiles provides unprecedented resolution. These results also resonate with
anecdotal observations of other differences, such as changes in splice site selection
during the course of intracellular growth (Fig 2.9).

![PCA – binned by time intracellular](image)

**Figure 3.4.** Spatial distributions of tachyzoite-specific gene expression patterns by PCA
analysis binned by time post-infection.
PCA for tachyzoite samples binned based on time post-infection.

**A role for opposite strand transcripts in regulating stage-specific expression?**

As noted above, in addition to protein-coding genes, long non-coding transcripts
(lncRNAs; Patil et al. 2013) are also observed *T. gondii* RNA-seq datasets, and circum-
stantial evidence suggests a possible role in transcriptional regulation. For example, the
location of tachyzoite-specific lncRNAs in Figs 2.3 & 2.11A (*green boxes*) suggests that
they may act to suppress (or degrade) full-length mRNAs on the opposite strand. A quick search of the *T. gondii* database for genes with abundant support for sense-strand transcription in tachyzoites and antisense transcription in gametocytes yields dozens of genes, including several AP2-family transcription factors (Walker, Gissot, Huot, et al. 2013; Walker, Gissot, Croken, et al. 2013; Grigg et al. 2001; Behnke et al. 2014; Hehl et al. 2015). As shown in Fig 3.5A, there is a strong inverse correlation between expression of *TgME49_282210* (AP2-VIIA8) mRNA in tachyzoites but not gametocytes, and opposite strand transcription in gametocytes but not tachyzoites. A similar inverse relationship was also observed for mRNA expression in bradyzoites and sporozoites, and lncRNA transcription in oocysts (not shown, but see Additional File 4). Neither stage-specific differences nor opposite strand transcripts were detected in the adjacent gene (*TgME49_282210*), which is also a putative AP2 transcription factor (AP2-VIIA9). Further inspection reveals a similar sense and antisense transcription patterns for many other genes in the parasite genome (Fig 3.5B).

To further explore the relationship between mRNAs and opposite strand lncRNA expression genome-wide, sense vs antisense transcript abundance was examined for various pairwise sample comparisons. Comparing tachyzoites with gametocyte samples (Fig 3.6) shows that the vast majority of genes are similarly expressed (central black cloud), consistent with observations in Table 2A. Some genes display stage-specific expression, with steady-state levels that differ by up to $10^5$-fold. Positive controls for tachyzoite-specific, gametocyte-specific, and constitutively expressed genes are indicated in red, purple and green, respectively). The size of datapoints are scaled to reflect maximum mRNA abundance for the comparisons shown (FPKM values), *i.e.* large dots reflect abundant transcripts (ribosomal RNAs are indicated in gray).
Figure 3.5. Selected examples of genes displaying an inverse stage-specific correlation between antisense RNA and mRNA.

**Top: Gametocyte vs Tachyzoites.** mRNA for TgME49_282210 (encoding AP2-VIIA8; left) is expressed on the reverse (red) strand in tachyzoites, while overlapping lncRNA is transcribed on the forward (blue) strand in gametocytes (see also reciprocal arrangement in Figs 2.3 & 2.11A). Note that the adjacent functionally-related gene (TgME49_282220 = AP2-VIIA9; at right) is constitutively expressed, with no antisense transcription. **Bottom: Oocysts vs Tachyzoites.** Tachyzoite-specific mRNA (TgME49_321590) and overlapping lncRNA on the reverse strand in gametocytes (left); the adjacent tachyzoite- and oocyst-specific mRNAs (TgME49_321570 & -321580, respectively) are convergently transcribed, with long overlapping 3'UTRs (right).
Figure 3.6. Antisense RNAs are inversely correlated with stage-specific mRNA transcript abundance during *T. gondii* differentiation.

The ratio of tachyzoite/gametocyte expression on the mRNA sense-strand (horizontal) vs antisense expression (vertical), for all annotated genes in the *T. gondii* genome. Points are scaled to reflect maximal mRNA abundance for the two samples represented in each graph; shading indicates excess antisense/sense strand expression in tachyzoites (orange) or gametocytes (blue); gray denotes ribosomal transcripts. For example, the yellow star at center/left corresponds to the transcription factor AP2-VIIA8 (*TgME49_288210*), which is abundantly transcribed on the sense strand in gametocytes, and overlaps a tachyzoite-specific lncRNA on the opposite strand (left part of Fig 3.5A); X’s indicate transcripts known to be constitutive (actin, alpha-tubulin1, histone H3.3, Asp1, calmodulin; green), or specific to tachyzoites (SAG1, GRA1, MIC1, ROP15, ENO2; red), gametocytes (SRS12A, SRS15A, SRS22B-1&2, SRS37B; purple).

Antisense transcription is generally less abundant than mRNA (cf. Fig 2.11), and less variable between samples, but differences of up to $10^3$-fold are observed for some genes. In gametocytes, these is a slight correlation between sense and antisense expression, resulting in a positive overall slope, especially in the right half of Fig 3.6.
Shading reflects an overabundance of antisense transcript relative to the mRNA, i.e. tachyzoite-specific mRNA transcripts associated with gametocyte-specific antisense transcription (such as TgME49_282210; Fig 3.5A) are shaded blue.

Conversely, many gametocyte-specific genes (at right) are transcribed on the opposite strand in tachyzoites (orange shading). These differences are even more evident in comparisons of tachyzoites with parasite oocysts (Fig 3.7). More genes are differentially transcribed in tachyzoites vs unsporulated oocysts, and a slightly wider range of antisense transcript ratios is also observed (there was no discernable correlation between sense and antisense transcription within these tachyzoite or oocyst experiments). More tachyzoite-specific genes display excess antisense transcription in oocysts, and the majority of unsporulated oocyst-specific transcripts are correlated with tachyzoite-specific antisense transcription. Three intriguing examples are highlighted in Fig 3.5B: TgME49_321590 (an uncharacterized ‘hypothetical protein’) is transcribed on the mRNA (sense, blue) strand in tachyzoites (as well as bradyzoites & gametocytes; not shown), and on the opposite (antisense, red) strand in unsporulated oocysts (and sporozoites; not shown). The adjacent genes TgME49_321580 (a putative membrane protein) and TgME49_321570 (FabZ) are also stage-specifically expressed, in either unsporulated oocysts only (TgME49_321580) or tachyzoites (TgME49_321570). Interestingly, these genes are convergently transcribed, with 3’ UTRs that overlap extensively, providing the potential for antisense-mediated regulation based on mRNAs rather than IncRNAs.
Figure 3.7. Antisense RNAs are inversely correlated with stage-specific mRNA abundance during *T. gondii* differentiation. (A) Tachyzoites vs unsporulated oocysts, (B) d4 sporulated oocysts, (C) d10 sporulated oocysts. Points are scaled to reflect maximal mRNA abundance for the two samples represented in each graph; shading indicates excess antisense/sense strand expression in tachyzoites (orange) or oocysts (blue); gray denotes ribosomal transcripts. For example, the yellow star at far right in panel A corresponds to Oocyst Wall Protein 2 (*Tg*ME49_209610), which is abundantly transcribed on the sense strand in oocysts, and overlaps a tachyzoite-specific IncRNA on the opposite strand; other yellow stars correspond to the three genes in Fig 3.5B. ‘X’s indicate transcripts known to be constitutive (actin, alpha-tubulin1, histone H3.3, Asp1, calmodulin; green), or specific to tachyzoites (SAG1, GRA1, MIC1, ROP15, ENO2; red), oocysts (OWP2, TBPIP, RAD54, *Tg*ME49_249520 & 263010; blue), or sporozoites (MIC13, SRS28, *Tg*ME49_209920, 259900 & 276880; turquoise).
Samples isolated after the induction of sporulation provide the opportunity to follow changes in sense and antisense transcript abundance during differentiation. Day 4 sporozoites (Fig 3.7B) are much more similar to tachyzoites, and day 10 sporozoites even more so (Fig 3.7C). *i.e.* the cloud of transcription ratios becomes progressively tighter. Importantly, antisense transcripts also change in parallel with this change in transcriptional profile, as oocysts differentiate into the sporozoites that will transform into tachyzoites once they infect a host cell: orange dots become less prominent, as oocyst genes (with tachyzoite-specific antisense transcripts) are downregulated, and the blue dots essentially vanish, as the antisense RNAs to tachyzoite-specific mRNAs that were abundant in oocysts disappear during the course of sporozoite differentiation.

**Discussion**

At least 69 RNA-seq samples are currently available for *T. gondii* parasites, many of which are described here for the first time (Table 1). Considered in their entirety, these studies sample strain diversity (Hassan et al. 2012), the intracellular tachyzoite replicative time course, and most major life cycle stages. Expression profiling has been used to investigate developmental signatures in many systems (Uhlén et al. 2015), and it is not surprising that major developmental stages can readily be distinguished (Table 3 & Figs 3.1 -3.4). Results obtained from these analyses are consistent with previous analyses of individual genes, and array-based transcriptional profiling (Fritz, Buchholz, et al. 2012; Wasmuth et al. 2012; Buchholz et al. 2011; Radke et al. 2005; Bahl et al. 2010). Given their substantial biological differences, it is gratifying to observe that differences between life cycle stages are much larger than differences between strains (Fig 3.2). The relatively low cost of RNA-seq allows analysis of multiple samples, including time
course studies, and the high degree of resolution provided by strand-specific RNA-seq identifies reproducible differences even within the 48 hr intracellular tachyzoite replicative cycle, which has not previously been reported (Fig 3.3, Fig S6). In this regard, it is interesting to observe consistent differences in transcript splicing (Fig 2.9B, Fig S6), although the biological significance (if any) of these isoforms is not known.

It is also clear that transcripts need not be restricted to mRNAs, as hundreds of lncRNAs are evident in the *T. gondii* genome, often overlapping stage-specific mRNAs, and reciprocally expressed (Figs 2.3, 2.11A & 3.5). Some lncRNAs span the opposite strand gene (Fig 3.5), while others cover only a small portion (Figs 2.3 & 2.11A). In other cases, stage-specific expression of convergently-transcribed genes yields long 3'UTRs providing antisense coverage (Fig 3.5B, *right*). Inversely correlated sense and antisense transcripts are most prominent in comparisons of tachyzoites with oocysts (Fig 3.7), followed by tachyzoites vs gametocytes (Fig 3.6). There is little evidence for antisense association with the developmental transition from tachyzoites to bradyzoites (not shown, but see Additional File 4). The inverse correlation between sense and antisense RNA abundance suggests a causal relationship, and it is tempting to rationalize these observations by imagining that the tight regulation of transition to gametocytes (in the cat gut), and oocysts (whose metabolism is very different, and must survive for months in the harsh external environment), could explain the use of non-transcriptional mechanisms for silencing. Evaluating such hypotheses will require experimental testing, ideally using an *in vitro* model of the coccidian cycle in the definitive host.

The nature of lncRNAs is not entirely clear in *T. gondii* and other apicomplexan parasites: PCR amplification of putative lncRNAs has not always been reliable, suggest-
ing the possibility of multiple shorter transcripts rather than one long transcript; upstream sequences are not always effective as promoters in reporter assays; IncRNAs are not generally associated with active or repressive chromatin marks; and it is not even certain that *T. gondii* IncRNAs are all polyadenylated Pol II transcripts. *T. gondii* does possess Argonaut and Dicer proteins, but it is not clear that these mediate silencing (Meissner et al. 2007; Crater et al. 2016). It would be very helpful to have more effective methods for IncRNA prediction, but such algorithms have proved difficult to develop, and are not broadly applicable across diverse species (Fiscon, Paci, and Iannello 2015; Liao et al. 2011). By definition, coding potential is not relevant for assessing non-coding RNAs, and many IncRNAs display evidence of multiple introns that are very inefficiently excised (*cf.* Fig 3.5).
CHAPTER 4: SUMMARY, GENERAL DISCUSSION AND FUTURE DIRECTIONS

Structural annotation of gene models has been a challenge since the early days of genome sequencing, and has engaged both molecular biologists using experimental methods (RT-PCR, EST sequencing) and computational biologists responsible for ab initio and de novo methods. Deep sequencing of RNA molecules has revolutionized gene prediction, as it is now possible to obtain hundreds of millions of short RNA (or polyA+ mRNA) reads from individual samples. RNA-seq reveals an immense number of splice junctions, however, making it difficult to distinguish functional mRNAs from splicing machinery errors and experimental artifacts. Distinguishing alternatively spliced isoforms that are likely to be functional and should be annotated, from those that should not, is an important unsolved problem.

In order to study the prevalence of alternative splicing, we analyzed transcript expression from all available RNA sequencing experiments for the protozoan parasite Toxoplasma gondii, which displays typical eukaryotic splicing. This species’ genome is substantially smaller than that of humans and most common model organisms: ~65Mb, encoding 8322 protein coding genes, with 40,103 annotated introns. Several alternatively-spliced genes have previously been characterized in T. gondii, including examples of intron retention, exon-skip polymorphisms, and splice acceptor and donor variants … providing positive controls for this analysis (Fig. 2.1). There is also good reason to believe that algorithms used to identify alternative splice products in T. gondii will be applicable to other systems, as human nuclear extracts can properly splice T. gondii primary mRNAs, and vice versa.
Gene annotation

Taken together, this work outlines effective strategies for exploiting RNA-seq data-sets to improve the annotation of draft eukaryotic genomes. New RNA sequencing technologies provide high depth, high quality transcriptomic evidence from entire genome(s), which has proven to be extremely valuable for improving annotation, and revealing unappreciated transcripts (Djebali et al. 2012; Harrow et al. 2012; Mudge and Harrow 2016).

The aggregate pool of RNA-seq data for all samples initially considered in this study (Table 1) provides considerable information: the observed total of >2.7 million reproducibly-observed introns expands the number of T. gondii transcripts by many fold. Most of these junctions are exceedingly rare, however, as they were observed fewer than six times in the total of 69 possible samples. Furthermore, many of these rare junctions usually lie in non-coding regions such as UTRs (Fig 2.1) and IncRNAs and this is not surprising as higher sequence variation is known to be present in less conserved non-coding sequences than in coding sequences (Mu et al. 2011; Castle 2011).

The current T. gondii genome annotation was used as a guide to define parameters that minimize false discovery of transcripts. Exploiting the relationship between the abundance of reads that span introns or ISRPM and the reads that map to annotated genes or FPKM (to which the introns map to), provided good separation between True Positive (black datapoints in upper half of Fig. 2.6) and True Negative introns (black numbers in the right-hand column of Table 2A), even when applied to other life cycle stages (Table 2A and Fig 2.7A) and other species (Neospora, Plasmodium; same parameters were applied to genomes in EuPathDB.org). Introns with low ISRPM/FPKM are
inefficiently spliced and can be excluded from further analysis even if they are frequently observed in heavily transcribed genes. An even better strategy is to directly compare intron abundance to the number of reads in the flanking exons of each intron, instead of using the expression of the entire gene to which the intron maps to. Several other methods use ψ or Percent Spliced In to study the prevalence of alternative splicing across transcriptomes (Venables et al. 2008; Katz et al. 2010; Shen et al. 2014; Trapnell et al. 2013; Vaquero-Garcia et al. 2016). These alternative methods are aware of the change in coverage that can occur in a gene, if the gene is misannotated for example, and that is usually masked out by the fact that the total number of reads that map to a gene are normalized by the length of the gene, even if the coverage is not uniform in different exons of the same gene. Despite these differences, the method described here serves as an adequate heuristic when existing genome annotation is close to accurate. This method is also relatively straightforward to implement, as it is relatively straightforward to automatically extract from RNA-seq mapping pipelines and genome databases. This method could be easily applied to other organisms for which similar databases are available.

We found that best proportion of TP to TN introns occurred when we considered the maximal ISRPM from the 20 high quality samples selected in this study (Table 1) and compared it to the FPKM of the same sample where the maximal ISRPM was expressed (Table 2A and Fig 2.7B). This analysis showed that most False Positive introns individually analyzed in different samples turned out not be real FP introns: many were stage-specifically expressed (Fig 2.7B) and some were real alternatively-spliced isoforms (Fig 2.8). Likewise numerous False Negative introns were not completely false as they were
alternatively-spliced, but were less abundant than the annotated TP alternative (Table 2B).

In summary, we show that >93% of ~40K annotated introns associated with ~9K genes in the reference genome are strongly supported by RNA-seq data from at least one sample, leaving ~5% that should be deleted, and 2% that should be supplanted by alternative introns. In addition, we describe ~20% well-supported new introns, including 4% that are alternatively-spliced (many within UTRs), ~8% that modify existing gene models, and ~8% associated with novel transcripts (approximately equally divided between predicted mRNAs and lncRNAs). Abundant introns associated with novel transcripts represent either genes that are specifically expressed in previously under-studied stages (cf. Figure 2.5) or lncRNAs whose exact function is not known, but thought likely to be involved in transcriptional regulation (cf. Figs. 3.5-7). One of the difficulties in the current annotation is the definition of UTR boundaries, as the algorithms that were used for calling UTRs applied RNA-seq data in a greedy manner. Instead of using the most abundant transcript to define UTR boundaries, the algorithm incorporated information from the longest possible transcript. This means that a portion of annotated gene models have potentially long UTRs, sometimes affecting correct call of transcriptional and/or translational start sites (cf. Figs 2.7-9). Usage of the most abundant transcript from RNA-seq data instead of the longest, should correct this problem in principle. However, as shown in Figure 2.8, this solution is difficult to implement as a general rule in all annotated genes. For example, in the case of Isocitrate Dehydrogenase (Fig 2.8) it would result in exclusion of the first annotated intron, which is clearly excised more frequently in a stage-specific manner in tachyzoite parasites. This intron is less often excised in the cat enteroepithelial stages because there is a stronger promoter within
this intron that would preclude excision most of the time. It is difficult to decide which transcripts should be annotated and which ones should not.

Genome annotation and selected RNA-seq tracks from gametocytes and late trophozoites. Note that blue and red tracks are scaled logarithmically, while tracks shown in semi-transparent overlay are linear (note different scales, selected to highlight stage specific expression). Arrows and circles indicate transcript termini. Note the two translational starts in A (arrowheads), which have been confirmed to have different localizations within the strain 3D7 of *P. falciparum* (Kehr et al. 2010). Two tracks display candidate introns (brackets): the ‘filtered’ track is restricted to display only those introns that pass the abundance and efficiency criteria described in this dissertation. The star in panel B indicates an acceptor splice variant that deletes a single aminoacid from the Rifin transcript.

**Figure 4.1.** Application of ISRPM/FPKM parameters to *Plasmodium* RNA-seq datasets discriminates significant introns from low abundance variants (from PlasmoDB.org).
Most biologically-significant errors in the current reference annotation for *T. gondii* relate to inaccurate annotation of transcript initiation, often impacting the predicted translational start site (Figs. 2.7 & 8), and hence subcellular localization predictions (which are particularly important for pathogenic species that secrete factors modifying the host response). These kinds of errors can be addressed by several mechanisms, such as implementing known features of transcriptional initiation context (Matrajt et al. 2004), incorporating evidence from conserved sequences in orthologous genes and proteins from other related species as well as evidence from proteomic experiments. Other solutions could also be to consider data from promoter binding experiments (Niu, Tabari, and Su 2014) as well as from full length RNA-seq (Sharon et al. 2013) and other sequencing technologies that sequence fragments of up to 150kb (Jain et al. 2016).

Longer reads will also benefit research and analysis of alternative splice variants, as complete or near complete sequenced RNAs would have less sources of error generally produced during the assembly of short reads from current Illumina NGS into transcript(s). In order to estimate the significance of alternative splice variants, phylogenetic comparisons aiming to ask if putative variants are conserved in several strains of *T. gondii* or in other closely related species, such as *Neospora sp.* and *Plasmodium sp.* should be considered. Examination by multiple sequence alignments of how different regions within potential variants change in sequence in their ortholog counterparts should provide more evidence towards the functional significance, if any, as well as the conservation of these variants among several related species. Characterization of changes in abundance of alternative transcripts across different spliceosomal mutants
should provide an extra layer to evidence about the potential biological significance of splice variants.

The existing genome annotation could also benefit from identifying new unannotated transcripts such as IncRNAs. Our current ability to predict plausible IncRNAs from sequencing data is poorly developed, as definition of IncRNA transcripts has proven difficult because they do not contain the same sequence features as protein coding transcripts. Developing new gene finders that use machine learning techniques to learn sequence features from known *T. gondii* IncRNAs would be the best approach to characterize IncRNAs, as methods developed for other species have proven not applicable in *T. gondii* since they are specific for the system for which they were developed (Liao et al. 2011; Fiscon, Paci, and Iannello 2015).

**Transcriptional regulation**

In order to study possible mechanisms of transcriptional regulation, we also carried out transcriptional profiling by RNA-seq, including other available transcriptional data to encompass sequencing information from all stages of the complex parasite life cycle (Table 1). Steady-state transcript levels highlight important differences, even within the intracellular replication cycle, and circumstantial evidence suggests an important role of antisense transcription (including IncRNAs) in regulating stage-specific expression. Stage-specific expression was previously known is readily distinguished in all major life cycle stages by PCA and MDS analysis (*cf.* Figs. 3.1-3). As expected, between-stage differences were greater than between-strain differences (Fig 3.2), and to our knowledge, this report provides the first observation of profiling differences within the tachyzoite replicative cycle (Fig 3.4), including differences between intracellular and
extracellular parasites, and early and late stage intracellular tachyzoites. It will be interesting to determine the functional significance of these differentially-regulated transcripts within the tachyzoite lytic stage.

Analysis of strand-specific RNA-seq data has identified a previously unappreciated diversity of (unannotated) long non-coding RNAs (lncRNAs). We looked for differential expression of sense vs antisense transcripts in tachyzoites (the acutely lytic stage causing disease, which is readily cultivated) vs either bradyzoites (the latent cyst stage, which is less readily grown in culture), gametocytes (tachyzoites or bradyzoites undergoing sexual differentiation in the epithelium of the cat's gut) or sporozoites (the result of sexual crosses, which is not readily obtained in the lab) to examine elucidate the relationship between sense and antisense transcripts. The function of these transcripts is not yet clear, but many overlap the 3’ UTR of coding genes, and display stage-specific mutually-exclusive coding vs non-coding transcript expression (cf. Figs. 3.5, 3.6 & 3.7), suggesting a possible role in the regulation of stage-specific expression, specifically during the sexual stages.

In order to determine if antisense transcripts play a significant role in transcriptional regulation during parasite biology, several strategies and experiments could be carried in future research. One would be to map antisense lncRNAs promoters in T. gondii tachyzoites using for example a luciferase transient transfection assay. Other experimental option to elucidate the function of antisense transcriptional regulation would be to test the hypothesis that antisense lncRNAs regulate expression of sense overlapping mRNA transcripts, by manipulating their expression in transgenic parasites. Three experimental approaches could be used to test this hypothesis. One would be using the Ku80 knock-
out system for targeted homologous recombination in *T. gondii* to delete the 5’ upstream region (and ideally the promoter region) for tachyzoite-specific lncRNAs. Antisense and sense transcript expression will then be assessed by strand-specific qPCR. A second experimental strategy would be to overexpress antisense lncRNAs in tachyzoites from a tet-inducible promoter, following antisense and sense transcript expression/repression by strand-specific qPCR. Finally, a third experimental approach could exploit CRISPR mutagenesis to specifically change the sequence of strand-specific antisense transcripts and or their promoters in tachyzoite parasites and then monitor antisense and sense transcript expression/repression by strand-specific qPCR. These strategies, however, would generate fewer insights into a possible biological role of antisense transcripts because the most striking regulation appears to be occurring in the sexual stages of the parasite, especially sporozoites, where manipulation has proven to be difficult.

Other interesting projects could be continued from the data generated for this thesis. Sequencing experiments also yielded data on host cell responses to parasite infection as well as on small non-codingRNAs (sncRNAs) from the parasite and its host cell. Analysis of host cell responses to parasite infection should permit identification of co-regulatory patterns correlating host and pathogen gene expression, such as relationships between metabolic pathways and receptor-ligand interactions, among others. In related work, we also provided Jonathan Wastling’s group at Liverpool with parallel samples for quantitative proteomic analysis, and it will be interesting to compare parasite and host transcriptomics vs proteomics.

Mapping of sncRNAs reads to the parasite genome showed that they are most abundantly found on repeats and introns of genes (mRNA). Parasite expressed
sncRNAs are of several different sizes, varying from the 21-23nts size that are usually observed in humans. Different classes of sncRNAs, regulated in a tissue and time specific manner in plants and animals, are well known to play important roles in mechanisms such as defense, development and chromatin regulation (Kim and Sung 2012; O’Connell, Rao, and Baltimore 2012). However the apparent lack of an abundant unified size class (>50%) in T. gondii sncRNAs, indicates that the origin and possible function of sncRNAs is different from what is known in other organisms. The T. gondii genome codes for a Dicer and an Argonaute protein. The TgDicer protein, in charge of cleaving long dsRNA and generating siRNA or miRNA precursors, is composed of a RNA helicase domain and two RNase II catalytic domains. Nevertheless it lacks the conserved dsRNA binding domains, DSRM and PAZ, features also seen in the Dicer of the single cell Algae, Chlamydomonas reinhardtii. The TgArgonaute protein, displays conserved PAZ and PIWI domains, along with RGG amino terminal repeats (3X) which have the potential to be methylated, a characteristic shared with metazoan and plant Argonautes (Braun et al. 2010). T. gondii also possesses one representative of a RNA dependent RNA polymerase (RdRP) which is implicated in other organisms of amplifying sncRNAs from endogenous transcripts (Nishikura 2001). Modified tachyzoite parasites have been generated to contain a KO version of Argonaute, but standard characterization experiments don’t seem to show any defect, so it is not clear what function TgArgonaute is playing. To assess the possible function of sncRNA in T. gondii, it would be interesting to generate KO sexual parasites lacking each of the representatives possibly involved in small ncRNA biogenesis and test if they have any effect in the biogenesis and/or stability of small ncRNAs.
Human small ncRNAs were on average 23nt long, and mapped to several locations throughout the human genome, being most abundant in repetitive regions. The specific size of 23nt agrees with known animal miRs and siRNA sizes (McManus and Sharp 2002). Out of 317 human miRs analyzed, there were 164 (51.7%) that were suppressed two fold or more during infection with both TgRH and TgME49. Only 18 (5.7%) of human miRs were upregulated two fold or more during infection with these two strains, among which we found and confirmed what has been previously reported: upregulation of members of the family miR-17 (Zeiner et al. 2010). Additionally we have also encountered that human miRs -146a and -155 are also among the upregulated microRNAs. These have been reported to mediate a negative feedback loop regulation of the human transcription factor NF-κB (Taganov et al. 2006), which during T. gondii infection is known to be activated in varying degrees by different strains (Rosowski et al. 2011).

Examining Toxoplasma-infected human cells, we have now found that the more notable impact of parasite infection is a profound suppression of most human miR expression. Human miRs appear to be suppressed by infection with T. gondii strains RH and ME49, but not VEG and by N. caninum. It will be interesting to following up on these preliminary results, as they promise to reveal mechanisms of host-parasite interactions.

In summary, exploiting multiple RNA-seq datasets, from diverse sources, and integrating this information with other functional genomics data, permits substantial improvement in the annotation of the Toxoplasma gondii genome and transcriptome. The methods described here should be readily incorporated into computational pipelines, and are likely to prove useful for other species as well.
APPENDIX MATERIALS

The following supplementary materials are available in the supplemented CD:

**Additional File 1:** Microsoft Excel .xlsx file containing the following spreadsheets, each represented as a separate tab: Data, Stats, Annotated noEvidence (FP), Unannotated AltSplicing (FN) and Unannotated NewGenes (FN). ‘Data’ lists all annotated introns and unannotated intron-spanning reads observed at plausible abundance (59,755 rows), along with information on chromosomal position & strand, gene mapping & location within the gene, annotation status, ISRPM values, FPKM (when mapped to genes), and ISRPM/FPKM values, for all 20 high quality strand-specific RNA-seq datasets (see Table 1). Additional columns indicate the maximum ISRPM observed for each intron, the maximum ISRPM for any alternative overlapping intron, and corresponding FPKM & ISRPM/FPKM values. Appropriate columns from this table were used to generate Figs 2.4, 2.5, 2.6, 2.12 & 2.13. The ‘Stats’ tab provides counts and several statistics relating to the ‘Data’ tab, including data presented in Table 2. Other tabs present filtered views of ‘Data’ highlighting specific corrections recommended for the reference *T. gondii* genome annotation.

**Additional File 2:** PDF file containing supplementary figures S1-S5. Legends are included at the beginning of this file.

**Additional File 3:** Excel .xlsx file containing supplementary spreadsheets, presenting FPKM values for all 8920 annotated genes, from all 20 high-quality strand-specific
studies analyzed in this manuscript (Table 1), in addition to various averaged datasets, e.g. all strain ME49 parasites, all 4 hr post-infection samples, etc). Additional tabs present analysis of these data using various transcript abundance cut-offs, as noted, by PCA & MDS (see Figs 3.1, 3.2, 3.3 & 3.4 and Table 3), as well as HC (heatmap), AGC, etc (see Methods).

**Additional File 4:** Excel .xlsx file containing supplementary spreadsheets, presenting sense and antisense FPKM values for all 8920 annotated genes, from 20 high-quality strand-specific studies analyzed in this thesis (Table 1), plus the ME49 hr 2 sample, in addition to various averaged datasets, e.g. all gametocyte samples, and pairwise comparisons between samples e.g. the ME49 tachyzoite vs d7 CZ-H3 gametocyte comparison (presented in Fig 3.6). The second tab contains transformations of minimum and maximum FPKM values as indicated in the tab name.
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