**Dual host-parasite transcriptomes of apicomplexan *Eimeria falciformis* and its natural mouse host**

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**ABSTRACT**

Apicomplexan parasites such as Plasmodium spp., Toxoplasma gondii and Eimeria spp. cause disease in humans, livestock and wild animals. The genus Eimeria comprises >1800 niche specific intracellular parasites, most prominent among them several species which cause losses in poultry industries. Eimeria falciformis naturally infects the cecum of mice. Infecting one of the best studied and available animal models in biological research, this parasite can be used as a model to investigate Eimeria infections. However, much is still unknown about the parasite’s basic biology and no in vitro culture has been established for the full life cycle. We have performed a dual RNA-seq transcriptome study of the full life cycle in the mouse and of in vitro cultured sporozoites and oocysts. Drastic differences are seen in both parasite and host at three time-points post infection. Comparisons between immunocompetent and immunocompromised mice show differences in oocyst output as well as mouse transcriptional differences indicated by enriched functional Gene Ontology, GO, categories. In mouse, TGF-beta, EGF, TNF and IL-1 and IL-6 are examples of genes reacting differently depending on mouse immune status. Much in contrast, parasite transcriptomes are neither different between immune competent and immune deficient mice, nor between naïve and challenge infected mice. Instead, parasite transcriptomes have distinct profiles early and late in infection, characterized largely by biosynthesis and motility, respectively. Sporozoites and oocysts can also be identified by distinct transcriptional profiles. The use of hosts with different immune competence highlights the role of adaptive and innate immunity in the host and offers a source for in-depth analysis of these responses. Different life stage parasite transcriptomes from the natural host improves knowledge of the parasite biology and helps positioning E. falciformis as a model for Eimeria parasites in a scientifically valuable host animal.

Keywords

Parasite, apicomplexa, RNA-seq, transcriptome, life-cycle, interaction

INTRODUCTION

Eimeria falciformis is an intracellular parasite in the phylum Apicomplexa. Among more than 4000 described species of Apicomplexa (Duszynski 2011) prominent pathogens of human are found such as Toxoplasma gondii, causative agent of toxoplasmosis, Plasmodium spp., causing malaria, and Cryptosporidium spp, which cause cryptosporidosis. Coccidiosis is a disease in livestock and wildlife caused by coccidian parasites which are dominated by > 1800 species of Eimeria (Duszynski 2011). The genus is best known for several species which are problematic for poultry industry (Chapman et al., 2013). A useful model for studying Eimeria spp. is E. falciformis, which naturally infects wild and laboratory mice, Mus musculus (Heitlinger et al. 2014), with easy access to a broad range of genetically manipulated hosts.

Eimeria spp. infect the gut and are highly niche specific. E. falciformis has its niche in the caecum and upper part of colon, mainly in the cells of the crypts (Haberkorn 1970, Schmid et al., 2012; Stange et al 2009). These monoxenous parasites go through asexual (schizogony) and sexual reproduction which results in hosts releasing high numbers of resistant oocysts. When a mouse ingests E. falciformis oocysts, one sporulated oocyst releases eight infective sporozoites inside the host, which can infect epithelial crypt cells of the caecum and colon. Within the epithelium, so called merozoite stages form in several rounds of schizogony. Parasite numbers increase drastically during schizogony, which is not completely synchronized, and the exact number of schizogony cycles is either not clear (Haberkorn 1970, (Mesfin and Bellamy 1978) or it varies naturally. Haberkorn (1970) reported variation from one to four rounds of schizogony before gamete formation whereas Mesfin and Bellamy (1978) detected four distinct schizont stages. It therefore appears that the number and exact timing of schizont formation can vary in E. falciformis infections. Oocysts form as merozoites and differentiate into gametes, which fuse and form a zygote. Oocysts are first detected in faeces on day six to seven post infection, post infection, and output peaks on day eight to nine post infection After day 13-15 post infection oocysts are no longer detectable (Haberkorn 1970 and this study). Immature, unsporulated oocysts are shed into the environment where they mature into sporulated, infective oocysts (Mesfin and Bellamy 1978).

It is well known that Eimeria spp. generally induce protection against reinfection in hosts (M. Elaine Rose 1974; Mesfin and Bellamy 1979; Blagburn and Todd 1984; M. E. Rose, Hesketh, and Wakelin 1992; Gadde et al. 2009; Sühwold et al. 2010;) and that T-cell seem to play a major role. In host responses to E. falciformis infection of laboratory mice, IFNγ is upregulated (Schmid 2014). It was however shown that even though IFNγ and its receptor influences parasite development, the pathway is not required for clearance of a first infection, or for development of protective immunity in challenge infections. In the IFNγ deficient model which displays larger weight losses and intestinal pathology but also lower oocyst output, the phenotype was recovered by blocking IL-17A and IL-22 signaling. Only blocking IL-22 signaling recovered the oocyst shedding but had no effect on weight loss (Stange et al. 2012). These studies demonstrate the complex relationships between parasite and host and exemplify that larger pathology in the host is not necessarily beneficial for the parasite. Adaptive immunity clearly plays a role in limiting Eimeria spp. reinfection, but the effect on the parasite remains poorly understood. The closely related apicomplexan parasite T. gondii also induces immunity in its definitive host, which only sheds oocysts once in their life (VanWormer et al. 2013; Hartmann et al. 2013). Several genes which are identified as virulence factors in T. gondii are also present in Eimeria spp. genomes, such as surface antigen genes, SAG-genes and rhoptry proteins, ROPs (Heitlinger et al. 2014; Adam J. Reid et al. 2014). Although the number and functions of these genes are not identical, existing annotations of T. gondii orthologues are useful in studying Eimeria spp.. Additionally, the close relationship between the parasites and partly shared niche of these species, but the extreme difference in specificity between the generalist T. gondii and the highly specific Eimeria spp. makes comparisons evolutionarily interesting.

Several transcriptome studies address host parasite interactions in apicomplexan parasites (Periz et al. 2007; Aarthi et al. 2011; Schwarz et al. 2010; Amiruddin et al. 2012; Novaes et al. 2012; Adam James Reid and Berriman 2012; Schmid 2014; Matsubayashi et al. 2016) but often with a strong focus on either host or parasite. Reid and Berriman (Adam James Reid and Berriman 2012) have analyzed two microarray datasets from independent studies of Plasmodium and mouse, and mosquito, respectively (Lovegrove et al. 2006; Xu et al. 2005). In this metaanalysis, Reid and Berriman correlate host and parasite transcriptional changes to identify interacting host-parasite gene pairs and confirm the identified gene-interaction pairs by also analyzing protein domain information from which they infer direct host-parasite interactions at the molecular level. To our knowledge no transcriptome studies of apicomplexan parasites simultaneously and symmetrically asses both parasite and host over the full life cycle.

The genome sequences of seven Eimeria spp. infecting the chicken (Reid et al. 2014) and of E. falciformis (Heitlinger et al. 2014) lay the foundation for transcriptome studies based on high throughput sequencing of RNAs (RNAseq). Application to parasite research enables simultaneous assessment of RNA abundance (gene expression) in both host and parasite/pathogen, termed dual RNAseq (Foth et al. 2014; Rosani et al. 2015; Westermann et al. 2016; Fernandes et al. 2016; Li et al. 2016).

By applying such dual RNA-seq to produce such host and parasite transcriptomes from the same samples and tissue, we provide a first dataset which allows analysis of host and parasite mRNA profiles at several time-points post infection in an apicomplexan parasite. Not only can we for the first time provide transcriptomes for the full parasite life cycle of E. falciformis.

Repetedly reported resistance to reinfection with Eimeria spp. also makes it interesting to contrast these infections with parasitism in a host with strong deficiency in adaptive immune responses. In E. falciformis, nothing is known about parasite sensitivity to such differences in host immune status in different parasite life cycle stages. Also uninvestigated are potential genes that might interact with the host, and dual transcriptomes combined with established analysis methods (Adam James Reid and Berriman 2012) offer the possibility to evaluate such host parasite interactions.

RESULTS & DISCUSSION

**Effects of E. falciformis infection dynamics in immune competent and immune deficient mice**

To investigate E. falciformis development throughout the life cycle in its natural mouse host, dual transcriptomes were produced at days 3, 5, and 7 post infection. In contrast to wild-type mice, we also investigated parasite development and mouse transcriptomes in a mouse strain which is severely limited in adaptive immune responses (*Rag1-/-*) at day 5 post infection. To further elucidate host immune responses and parasite sensitivity to host immunity, we also challenge infected all mouse groups and sampled at the same time points as in naïve mice.

Basic phenotyping of wild-type mice showed drastically decreased in oocyst output (Figure 1 a) in challenged hosts compared to naïve animals. Similarly, a reduction in parasite 18S transcripts determined by quantitative reverse transcription PCR (RT qPCR) was detected (Figure 1b).

Therefore, as expected in Eimeria spp. infections, we demonstrate reduced intracellular parasite numbers (18S RT qPCR data) and reduced reproductive success (oocyst shedding) in immune competent, challenge infected mice. In contrast, no difference in reproductive success was observed between first and challenge infection in immune deficient mice. Oocyst numbers in faeces peaked on days 8-9 and all mice, also the immune deficient group, had cleared the infection by day 14. We thereby note that E. falciformis infection is self-limiting also in mice without mature T and B cells with the same kinetics as in immune competent mice. However, total reproductive success is reduced in immune competent mice only. Asexual replication and increase in parasite numbers of E. falciformis intestinal stages is reflected by the percentage of parasite material (reads) sequenced. This data is shown per time-point post infection (Figure 1c) and corresponds well with RT qPCR profile of 18S transcripts (Figure 1b). We thus use dual RNA-seq to analyze the life cycle of E. falciformis and host responses to infection. Transcriptomes infected hosts of different immune capacity at early and late stages of infection, and challenge infections are described.

*Parasite and host dual transcriptomes can be assessed in parallel*

We purified mRNA from infected caecum epithelium and demonstrate that even early in infection (3 days post infection) there is sufficient parasite material to detect the parasite mRNA among host mRNAs, and to quantify them by RNA-seq (Figure 1a). Total numbers of sequenced reads as well as reads mapped to either the E. falciformis genome or the mouse genome are indicated in Table 1 for all replicates. The number of total (host + parasite) read mappings for individual replicates ranged from 25,362,739 (sample Rag\_1stInf\_0dpi \_rep1) to 139,749,046 with 25% of the reads mapping to E. falciformis (NMRI\_1stInf\_5dpi\_rep3). Remarkably, on day 7 post infection, the day before oocyst shedding peaks, infected naïve mouse epithelium contained 77% and 92% parasite mRNA, i.e. more mRNA from the parasite than from the host (Figure 1c and Table 1). This can partly be due to transcriptionally active parasites and/or inactive/apoptotic host cells, but is likely due to high parasite numbers.

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| Sample\* | Sequencing method | Batch | Total reads | Reads mapping mouse | Reads mapping  *E. falciformis* | Percentage  *E. falciformis* | No.  *E.falciformis* genes |
| NMRI\_2ndInf\_0dpi \_rep1 | GAII | 2 | 108,937,797 | 70,489,674 | 247 | 0.0004 | 1 |
| Rag\_1stInf\_0dpi\_rep1 | hiseq | 3 | 25,362,793 | 18,853,850 | 443 | 0.0023 | 2 |
| C57BL6\_1stInf\_0dpi\_rep1 | hiseq | 3 | 35,731,249 | 25,119,348 | 457 | 0.0018 | 2 |
| C57BL6\_1stInf\_0dpi\_rep2 | hiseq | 3 | 47,085,959 | 34,377,133 | 608 | 0.0018 | 2 |
| Rag\_1stInf\_0dpi\_rep2 | hiseq | 3 | 46,556,156 | 35,233,327 | 676 | 0.0019 | 2 |
| NMRI\_2ndInf\_0dpi\_rep2 | hiseq | 3 | 58,122,244 | 40,794,245 | 3,406 | 0.0083 | 51 |
| NMRI\_2ndInf\_3dpi \_rep1 | hiseq | 3 | 57,934,016 | 40,544,287 | 4,803 | 0.0118 | 95 |
| NMRI\_2ndInf\_5dpi\_rep2 | hiseq | 3 | 63,965,539 | 48,289,181 | 10,941 | 0.0227 | 407 |
| NMRI\_1stInf\_0dpi\_rep1 | GAII | 1 | 82,364,585 | 55,176,243 | 17,954 | 0.0325 | 701 |
| NMRI\_2ndInf\_3dpi\_rep2 | hiseq | 3 | 65,548,826 | 46,171,909 | 29,548 | 0.0640 | 1,58 |
| NMRI\_2ndInf\_7dpi\_rep2 | hiseq | 3 | 67,487,466 | 51,722,265 | 40,091 | 0.0775 | 1,836 |
| Rag\_1stInf\_5dpi\_rep1 | hiseq | 3 | 38,651,359 | 29,982,453 | 63,024 | 0.2098 | 2,548 |
| Rag\_1stInf\_5dpi\_rep2 | hiseq | 3 | 34,779,832 | 25,297,803 | 99 | 0.3898 | 2,828 |
| C57BL6\_1stInf\_5dpi\_rep1 | hiseq | 3 | 40,904,388 | 29,319,604 | 185,969 | 0.6303 | 4,173 |
| Rag\_2ndInf\_5dpi\_rep1 | hiseq | 3 | 50,049,848 | 37,093,621 | 192,856 | 0.5172 | 4,167 |
| C57BL6\_1stInf\_5dpi\_rep2 | hiseq | 3 | 29,511,368 | 18,062,349 | 215,696 | 11.801 | 3,823 |
| C57BL6\_2ndInf\_5dpi\_rep1 | hiseq | 3 | 35,148,432 | 25,660,184 | 262,909 | 10.142 | 4,563 |
| NMRI\_1stInf\_3dpi\_rep1 | GAII | 1 | 73,236,430 | 49,993,358 | 394,384 | 0.7827 | 5,22 |
| NMRI\_1stInf\_3dpi\_rep2 | GAII | 2 | 160,709,694 | 117,791,044 | 413,051 | 0.3494 | 4,862 |
| NMRI\_1stInf\_5dpi\_rep2 | GAII | 2 | 119,902,722 | 76,419,774 | 794,57 | 10.290 | 5,333 |
| NMRI\_2ndInf\_5dpi\_rep1 | GAII | 2 | 230,773,955 | 143,186,486 | 1,846,840 | 12.734 | 5,533 |
| NMRI\_2ndInf\_7dpi\_rep1 | hiseq | 3 | 70,366,762 | 41,467,146 | 8,634,201 | 172.335 | 5,875 |
| NMRI\_1stInf\_5dpi\_rep1 | GAII | 2 | 76,702,168 | 47,037,087 | 8,669,701 | 155.631 | 5,7 |
| NMRI\_sporozoites\_rep2 | GAII | 0 | 19,551,681 | 8,656 | 11,470,604 | 999.246 | 5,513 |
| NMRI\_1stInf\_5dpi\_rep3 | GAII | 0 | 191,099,180 | 83,735,624 | 27,839,458 | 249.513 | 5,784 |
| NMRI\_1stInf\_7dpi\_rep1 | GAII | 1 | 66,505,514 | 3,310,666 | 39,400,884 | 922.488 | 5,932 |
| NMRI\_sporozoites\_rep1 | GAII | 1 | 67,325,397 | 4,334 | 43,774,401 | 999.901 | 5,825 |
| NMRI\_oocysts\_rep1 | GAII | 1 | 68,859,802 | 3,805 | 49,653,065 | 999.923 | 5,695 |
| NMRI\_oocysts\_rep2 | GAII | 0 | 151,090,783 | 18,524 | 71,019,860 | 999.739 | 5,777 |
| NMRI\_1stInf\_7dpi\_rep2 | GAII | 1 | 139,749,046 | 21,699,324 | 73,539,445 | 772.159 | 5,943 |

**The mouse transcriptome undergoes broad changes upon *E. falciformis* infection**

We here show that upon infection with E. falciformis, which induces weight loss and intestinal pathology in mice (SI xx, pathology data not shown), the host transcriptome undergoes drastic changes with more than 3000 genes changing their mRNA profile. Statistical testing for differential expression between infected and uninfected mice revealed changes in mRNA abundance becoming more pronounced (both in magnitude and number of genes affected) at later time-points post infection (Table 2 and Figure 2A). 325 mouse mRNAs were considered differently abundant (FDR < 0.01) between controls and 3 days post infection , 1,804 mRNAs between controls and 5 days post infection and 2,711 mRNAs between controls and 7 days post infection. This leads to a combined set of 3,453 unique genes responding to infection (Figure 2b). Differentially abundant mRNAs early in infection (3 and 5 days post infection) were not a strict subset of genes differentially abundant later in infection (7 days post infection), which would be the case if the same genes are regulated throughout infection.

Instead, the transcriptional profile of the mouse changes more fundamentally with other genes regulated late compared to early in infection. Our results for late infection are in agreement with previously published microarray data from mice infected with E. falciformis species (Schmid 2014), for which our 7 days post infection data was correlated. The two data-sets show a strong correlation (Spearman's rho = 0.74; SI XX). Considering both biological differences in the experiments such as exact time-points for sampling, and technical differences between the two methods, this correlation confirms the adequacy of using dual RNA-seq for assessing the host transcriptome.

To further analyze the distinct responses early and late in infection, we performed hierarchical clustering on the mouse genes differentially abundant between different time-points post infection (Figure 2c). Three main sample clusters formed (dendrogram of columns at top of Figure 2c). Immune deficient *Rag1-/-* mice, including infected samples, cluster with uninfected samples. The similarity between infected and non-infected *Rag1-/-* samples, confirms the immune deficiency phenotype (failure to react to infection) in these mice and suggest a strong influence of adaptive immune responses on overall transcriptional response. Surprisingly, these patterns indicate that innate immune responses and other B- and T- cell independent processes play a minor role in the host response.

***B and T cell dependent early responses to parasitic infection***

For immune competent hosts, clustering of genes and samples based on differentially abundant mRNAs (Figure 2b) clearly distinguishes non-infected controls from infected animals. On days 3 and 5 post infection, two clusters of genes have overall high mRNA abundance (1 and 2). They are also different from controls in having low mRNA abundance in two gene sets which are dominated by high mRNA abundance in non-infected controls (3 and 4). Among these genes which are lower in abundance upon infection, overrepresented GO terms are, e.g., “regulation of” the following: transforming growth factor-β, TGFβ, epidermal growth factor, EGF, tumor necrosis factor, TNF”, “negative regulation of interleukin-1 secretion”, “negative regulation of interleukin-6 secretion”, “Notch receptor processing”, “calcineurin-NFAT signaling cascade”, “Inositol-phosphate mediated signaling”, “autophagy”, “response to mechanical stimulus”, “ammonium transmembrane transport”, “positive regulation of axon guidance”, "lipid metabolic process", "protein complex localization" and "blood coagulation". These terms indicate an induction of IL-1, which is involved, e.g., in T and B cell maturation and IL-6, involved in, e.g., broad acute phase immune responses and T and B cell maturation (UniProt entry for Interleukin-1 alpha, and Interleukin-6, 29.11.16). TGFβ is important for wound healing in intestinal epithelium (beck03), and EGF regulates proliferation of epithelial cells and inhibits apoptosis (suzuki10). TNF is dose-dependent and apart from inducing several immune responses is also reported to regulate proliferation of epithelial cells (kaiser97). IL-6 has also been shown to support tissue repair and inhibit apoptosis after epithelial wounding (kuhn14), probably through the Janus kinase, JAK, and signal transducer and activator of transcription STAT3 (pickert09). IL-6 is also known to be important for development of Th17 responses (ref in kuhn intro) which play an important role in responses to E. falciformis (stange--). Inhibition of Notch signaling in mouse epithelium has been shown to alter the composition of cell-types in the epithelium towards Paneth and Goblet-like cells (VanDussen et al. 2012). Although speculative, several of the GO-terms in gene sets 1-4 can be linked: Inositol signaling can lead to release of calcium and calcineurin-dependent translocation of NFAT to the nucleus and activation of its target genes in T cells, but also many other cell types (reviewed by macian05). In addition, changes in the host epithelium do take place when cells are invaded by, e.g., E. falciformis, but also generally by pathogens. It is important to note that the specificity in the GO-terms alone is not enough to determine whether healing is induced or repressed (compare “regulation of” and “negative regulation of”). Further investigation of the role of the processes and molecules highlighted here will contribute to better understanding for responses to intestinal intracellular parasitic infection, intestinal wound healing and other epithelial remodeling and how it is regulated.

GO terms enriched among the mRNAs which become higher in abundance early in infection are, e.g., “stem cell population maintenance”, “mRNA processing”, and “cell cycle G2/M transition”, also indicating changes in the epithelium. In addition, terms such as “regulation of response to food” and “negative regulation of appetite” are enriched. A somewhat unexpected term, “photoreceptor cell development”, (and similar terms pointing towards visual perception) is supported partly be the gene mechanistic target of rapamycin, mTOR. mTOR is known as an amino acid sensor (e.g. Wellen and Thompson 2010; Laplante and Sabatini 2013; Gallinetti, Harputlugil, and Mitchell 2013) and it is therefore perceivable that it is involved in the regulation of food responses. Weight losses and malnutrition are generally common during parasitic infections (e.g. Preston-Mafham and Sykes 1970; Stephenson, Latham, and Ottesen 2000; Aloisio et al. 2006) and weight loss was also seen in this study (data not shown or SI file xx) in the intestine and these indications of responsible pathways and genes can be relevant to investigate in more detail.

Interestingly, in T- and B- cell deficient hosts, the same four groups of genes described above (1-4, figure 2c) show no differences between infected and non-infected animals. In two of these gene sets, all immune deficient mice - also infected ones - are most similar to non-infected immune competent control animals (1 and 4). These genes are the ones enriched for, e.g., regulation of IL-1 and IL-6, TGFβ, TNF, EGF, Notch receptor, and calcineurin-NFAT signaling (4) and, e.g., stem cell population maintenance, terms for cell cycle regulation and several RNA-processing related terms (1). In one gene set (3), all immune deficient animals’ mRNA profile is most similar to infected immune competent animals. Apparently, t- and B- cell deficient mice have similarities with infected wild-types for mRNAs linked to GO-terms for neuron differentiation and regulation and several metabolic processes. The pattern in gene set 3 suggests that these mRNAs have a high abundance in healthy wild-type animals in a T or B cell dependent fashion, since levels are low in T and B cell (maturation) deficient mice.

Innate immunity…. Expected but … (what about cytokines and Notch above?)

***Adaptive immune responses characterize late infection***

Pronounced transcriptional changes late in infection and GO terms enriched among those genes reflect the expected onset of an adaptive immune response (7 days post infection ; cluster 5 in Figure 2c). GO terms such as "antigen binding", "immunoglobulin receptor binding", "immune system process", "adaptive immune response", “cytokine production”, and also "innate immune response" and “regulation of apoptotic process” dominate this group of genes. Among the same genes, “natural killer cell regulation”, “neutrophil degranulation”, “JAK-STAT signaling”, and IL-1 and interleukin-2, IL-2 productions are enriched biological processes. Even on day 5 post infection, the genes responsible for these enrichments were low on mRNA abundance. This confirms a strong induction of immune responses, particularly adaptive immune responses between days 5 and 7 post infection. The result is well in line with previously described protective immunity against re-infection with *Eimeria* spp..

***Challenge infection…. something***

Three challenge infected samples from early and late secondary infection show a distinct mRNA profile (3, 5 and 7 days post infection, cluster 6, Figure 2b). These mRNAs are highly enriched for GO terms for RNA processing and splicing as well as terms for histone and chromatin modification. This suggests that protective immune responses in challenge infected animals are regulated both at the transcriptional and post-transcriptional level. The high abundance of these mRNAs at different time-points post infection in wild-type hosts also suggests individual variation in the timing of these responses. *Not sure how to conclude something here… Any thoughts? I did some literature research but with the search-terms used, did not get much wiser (eg “RNA regulation immune response” or “RNA regulation response parasite” on NCBI). Found some papers on cytokine/chemokine regulation by miRNAs in other diseases.*

Taken together, the mouse epithelial transcriptome changes upon infection by its natural parasite E. falciformis. The largest change is detected by comparing un-infected control animals to day 7 post infection samples, which is the day before oocyst shedding peaks. Early in infection, when the parasite reproduces asexually and multiplies within intestinal epithelial cells, in total ~750 unique mouse genes (days 3 and 5) are differentially abundant in their mRNAs compared to late infection. Late in infection ~1600 genes are uniquely differently abundant, i.e., mRNAs of these genes were not different early in infection. Sample clustering separates controls, early infection animals (days 3 and 5) and late infection animals from each other. Sample groups are characterized by specific mRNA abundance patters. By GO enrichment analysis for early infection, we identify regulation of a number of cytokines, stem cell population maintenance as wells as changes in food response or appetite. Late infection is dominated by terms which reflect adaptive immune responses and some which reflect innate immune responses. Responses seen in enriched GO terms in WT challenge infected hosts suggest strong gene expression regulation both at the transcriptional level and in RNA processing. In contrast, all these patterns are missing in T and B cell maturation deficient mice, and in general our analysis did not separate infected from un-infected immune deficient animals.

**Transcriptional differences in the parasite life cycle are independent of mouse immune status**

In the parasite transcriptome, we see no difference between infection in immune competent mice, T and B cell deficient *Rag1-/-* mice, or between naive and challenge infected mice. This is surprising considering the measured differences in oocyst output in the same comparisons (Figure 1a), and the fact that the same comparisons display distinct patterns in mouse transcriptomes. Major changes take place between 5 and 7 days post infection (Figure 3a, FDR < 0.01). Between 3 and 5 days post infection differences are smaller. Between early time-points, 3 and 5 days post infection, 103 mRNAs were different, whereas between 3 and 7 days post infection 1399 mRNAs were differentially abundant, and between 5 and 7 days post infection 2084 mRNAs were differentially abundant (Figure 3a). These results motivated us to define 3 and 5 days post infection as "early infection" and 7 days post infection as "late infection" also for the parasite transcriptomes. Early and late infection samples were tested for differential abundance compared to sporozoites and sporulated oocysts, resulting in 1697 and 3919 mRNAs, respectively. In order to find out more about the biological relevance of these patterns, we performed hierarchical clustering of the differentially abundant mRNAs described above and applied GO enrichment analysis of gene clusters based on annotations from Heitlinger et al. 2014. Distinct clusters of genes define early infection (3 and 5 days post infection, cluster 6) in which asexual reproduction takes place, and separately late infection (7 days post infection, clusters 2 and 7 ) in which we assume gametocytes to be present. Extracellular samples of sporozoites and oocysts are each defined by distinct gene clusters. Major patters in the parasite transcriptome seem to be determined by life cycle stages, and to be "transcriptionally blind" to the host immune status. Reinfection, resistance… Toxoplasma …

E. falciformis *unique genes in sporozoites*

Sporozoites are released from ingested oocysts in the gastrointestinal tract. We find that E. falciformis sporozoites are defined by a group of genes (cluster 4, Figure 3b and Additional file 5) which are largely specific to E. falciformis, meaning that few orthologues are found as gene family members outside of this species. Interestingly, five out of 12 SAG gene transcripts predicted for E. falciformis (Heitlinger 2012) are among the mRNAs highly abundant in sporozoites, suitable to the name “sporozoite surface antigens”, SAG. In total, 10 SAGs were detected as differentially abundant in our data. The five predicted SAGs present in sporozoites are orthologues of a SAG family member protein in chicken Eimeria spp. (EfaB\_MINUS\_56725.g2963), polynucleotide 5'-hydroxyl-kinase in T. gondii and hypothetical protein in chicken Eimeria spp. (EfaB\_MINUS\_7048.g636), and hypothetical proteins in chicken Eimeria spp. (EfaB\_PLUS\_25458.g2090, EfaB\_PLUS\_193.g6, EfaB\_PLUS\_3222.g332). SAG proteins are thought to be involved in host cell attachment and possibly in inducing immune responses in other apicomplexan species. The high enrichment of these mRNAs in an easily accessible life cycle stage opens for analysis of the function of these genes in E. falciformis. Other proteins which have received some attention in, e.g., E. tenella and T. gondii are rhoptry kinases, ROPs, which have been identified as important virulence factors. In E. falciformis sporozoites, two out of eight predicted ROPs were uniquely differentially abundant (EfaB\_PLUS\_8664.g829 and EfaB\_PLUS\_15899.g1411) which are orthologues of T. gondii ROP21 and ROP35 (the latter also in several chicken Eimeria spp.). These specific ROPs are commented on in more detail below, since they appear to have gene copies which are expressed also during asexual reproduction. In E. tenella sporozoites several ROPs are expressed and have also been shown to be differentially expressed compared to intracellular merozoite stages (Oakes et al. 2013). GO enrichment data on sporozoites suggests ATP production and biosynthesis processes as dominant features in sporozoites. Metabolic pathway analysis (ToxoDB) of the same genes reveals several pathways which also point towards ATP production, including fatty acid degradation, oxidative phosphorylation and valine, leucine and isoleucine degradation (Additional file 6). The latter degradation of branched chain amino acids pathway is enriched for genes which have orthologues in T. gondii, where they are used to utilize amino acids for energy supply (Oppenheim 2014). In addition, the invasive stage is characterized by "maintenance of protein location in cell" and similar GO terms. Possibly, this is due to control of microneme or rhoptry protein localization as sporozoites prepare for invasion. Sporozoites therefore display a transcriptome indicative of large requirements for ATP and production of known virulence factors SAG and ROP from closely related species.

*Growth processes dominate the transcriptome during asexual reproduction*

After sporozoites have invaded epithelial cells in caecum intracellular asexual reproduction can be initiated. Growth processes and the presence of several ROPs are characteristic for early infection, day 3 and 5 post infection. In early infection we expect several rounds of schizogony to take place in a somewhat unsynchronized fashion. Differentially abundant mRNAs cannot be distinguished between early 3 and 5 days post infection samples (cluster 6 in Figure 3b) which indeed indicates an unsynchronized parasite population. The same pattern is seen in mouse data, which suggests mutual influences between host and parasite. Among early infection mRNAs, several GO terms for biosynthetic activity are enriched, e.g., "ribosome biogenesis", "cellular biosynthetic process", "gene expression", "cellular amino acid catabolic process" and “RNA processing”, including terms for “tRNA and ncRNA processing”. Biosynthetic processes are enriched both in early infection and in invasive sporozoites, but the terms are driven by different genes/mRNAs. Analyzing conservation status in these lifecycle stage defining gene groups, sporozoite specific genes displayed a species specific profile (low number of orthologues outside E. falciformis), whereas early infection mRNAs are enriched for shared orthologues by 10 apicomplexan species (selected as in Heitlinger et al. 2014). This could reflect that similar processes and genes are involved in asexual reproduction among these apicomplexan species, whereas invasion may be a more species specific event. Among early infection high abundance mRNAs we found four out of 10 ROPs predicted in E. falciformis (Heitlinger et al. 2014). This is the largest number of ROPs in any group of differentially abundant genes in our analysis (EfaB\_PLUS\_24117.g1969, EfaB\_MINUS\_17096.g1521, EfaB\_MINUS\_42996.g2710 and EfaB\_PLUS\_7742.g778). The first, second and fifth of these have orthologues in T. gondii: ROP41, ROP35 and ROP21. In T. gondii type II, single deletions of ROP41 or ROP21 results in an intermediate decrease in cyst burden in mouse brain, compared to the parental Δku80 strain. The ROP35 deletion strain produced less, approximately a third, of the cysts compared to the parental strain. (Fox et al. 2016) However, the bioinformatically predicted ROP21 has been shown not to localize to the rhoptry organelles in T. gondii (Jones, Wang, and Sibley 2016) and might require re-annotation. Therefore, it is likely that this gene also in E. falciformis has functions which are different from rhoptry localized proteins. Enrichment of replication and growth-related processes highlights the parasite's expansion on 3 and 5 days post infection. This is supported both by previous knowledge about the life cycle and by the increase in parasite derived sequences we measured (Figure 1b and 1c). The mRNAs supporting growth related processes all have low abundance late in infection, on 7 days post infection. This likely reflects the switch from early asexual expansion towards differentiation and sexual reproduction on the time-point one day before oocyst output peaks. Apart from a clear transcriptional profile for growth, the presence of four out of 10 ROPs in the early intracellular phase suggests an important role for these proteins. Several ROPs (including ROP subsets known as rhoptry neck proteins, RONs) have been identified and partially characterized in other Eimeria spp. (Rick, Dubremetz, and Entzeroth 1998; Fetterer et al. 2013) and in T. gondii (reviewed in Bradley and Sibley 2007). Our data gives a first overview of expression patterns for E. falciformis ROPs and offer a good starting point for functional analysis of these virulence factors in mouse Eimeria spp.

*Gametocytes likely determine transcriptome late in infection*

Two E. falciformis gene clusters show a distinct profile characterized by high mRNA abundance on 7 days post infection (clusters 2 and 7). Both clusters display low mRNA abundance in other life cycle stages, especially in oocysts and sporozoites. Late stage defining mRNAs are enriched either for orthologues shared among core apicomplexan parasites (Apicomplexa excluding Cryptosporidium; cluster 2) or for genes conserved beyond the Apicomplexa (cluster 7, Additional file 5). Enriched GO terms such as "movement of cell or subcellular component" and "microtubule-based movement" along with terms suggesting ATP production ("ATP generation from ADP") indicate the presence of motile and energy demanding gametocytes in these samples. Other genes support ATP production in this life-cycle stage compared to sporozoites. For both gene groups that define late infection, different (peptide/nitrogen compound/cellular protein/macromolecule) "biosynthetic process" terms along with "chitin metabolic process" suggest that the parasite is producing building blocks for oocysts. This fits the timing of oocyst output which peaks at 8-9 days post infection , one day after sampling for RNA-seq. In addition, the Apicomplexa specific genes expressed in oocysts are enriched for a number of distinct GO terms such as "blood coagulation". These reflect the presence of Thrombospondin type I domains in the protein products of these mRNAs. Thrombospondin type I domains have been reported in E. tenella microneme localizing proteins, MIC, e.g. MIC4 (Tomley01). MIC4 mRNA was reported in E. tenella sporozoites where it localizes to the apical end, and in late schizonts and late oocyst stages, when sporozoites are forming. Possibly, the mouse parasite's MICs play a role in gamete stages or oocyst formation, or E. falciformis prepares for invasion already in during oocyst formation.

*Oocysts are characterized by stress responses and differentiation*

Oocysts are the infective stage in the parasite life cycle. They are shed with feces as unsporulated, “immature”, capsules and in the environment they undergo sporulation and become infective. Overall, the oocyst profile with five out of seven gene clusters characterized by below average abundance of mRNAs reflects the mainly inactive state of oocysts, which survive long times outside the host. However, two groups of mRNA are highly abundant, demonstrating that there is transcriptional activity in oocysts (clusters 1 and 5). One of the gene sets (cluster 5) is enriched for apicomplexan-shared orthologues and contains GO terms for "DNA repair", "protein modification process" and "cell differentiation". GO enrichment in the other group of genes (cluster 1, underrepresented for Eimeria spp. specific genes) highlights only one term (adj. P-value 0.11): "DNA-templated transcription, initiation". In our study, sporulated oocysts with ~90% purity were used. Sporulated oocysts are generally thought to be an inactive life stage. mRNAs which support GO enrichments for DNA replication and quality control might be a contribution from the ~10% unsporulated oocysts in the culture and would then reflect, e.g., DNA replication which takes place during sporulation. One candidate ROP was also detected in oocysts. mRNAs for which the annotated orthologue in T. gondii is ROP31 and in E. tenella ROP23 were highly abundant in oocysts but not present in any other cluster. Mice infected with T. gondii knock-outs for ROP31 had about half the amount of brain cysts compared to the control strain (Fox et al. 2016) but nothing is known about its influence on Eimeria spp. infections. Its presence in oocysts might indicate preparation for invasion, or suggest an unknown role in the oocyst stage. Our data on oocysts demonstrate that oocysts are transcriptionally inactive and that in the two clusters which do contain abundant transcripts, apicomplexan orthologues are enriched (5) or Eimeria spp. orthologues are underrepresented (1). Whereas underrepresentation can indicate a spread of conservational status among all other investigated categories and be difficult to interpret, the enrichment of apicomplexan orthologues in cluster 5 is more informative. Oocysts from many different apicomplexan species face similar challenges in the environment and can therefore be expected to share genes which are expressed in this life cycle stage. If such conservation can be confirmed, these genes constitute attractive targets for eliminating environmental oocysts in, e.g., farms.

*Evolutionary conservation in life cycle-characteristic gene groups*

A good understanding for similarities and differences between closely related parasites will be useful to draw parallels between species, and importantly also to avoid incorrect assumptions about, e.g., gene function. One way to achieve such understanding is to identify which genes that are species-specific, phylum specific or broadly conserved. In addition to the above described enrichment analysis of conserved orthologues in gene clusters (figure 3b) we performed Spearman's correlation analysis between our RNA-seq transcriptomes and RNA-seq data from related parasites. Two datasets for the economically important chicken parasite E. tenella (walker15 and reid14) and one dataset of the model apicomplexan parasite T. gondii (hehl15) were included in the comparison. For all samples from these studies and our data, transcriptomes were correlated and Spearman’s coefficient calculated (Figure…). This analysis confirms the species specificity for E. falciformis sporozoites. For Eimeria spp. transcriptomes samples from the same or similar life cycle stages tend to have the highest correlations with, e.g., E. tenella and E. falciformis gamete stages (gametocytes and late infection, respectively) correlating highly. However, E. falciformis sporozoites are more similar to other E. falciformis early infection samples than to orthologous sporozoite transcriptomes from E. tenella. The transcriptomes of E. tenella merozoites from both independent studies are most similar to early E. falciformis samples, indicating similarity also during asexual reproduction. The same result is shown by the conservation analysis of gene clusters in figure 3b (SI…) In the correlation analysis, E. falciformis oocysts cluster with unsporulated E. tenella oocysts, whereas E. tenella sporulated oocysts are most similar to E. tenella sporozoites, perhaps reflecting species specificity also in E. tenella sporozoites or possibly, differences in the in vitro preparation of these “environmental stages”. Our oocyst clustering with E. tenella unsporulated oocysts gives further support to the interpretation that our high abundance oocyst clusters (Figure 3b) are a result of transcripts contributed by the 10% unsporulated oocysts in our sequenced material.

The combined analyses highlight E. falciformis specific genes in sporozoites. Genes predominantly expressed in E. falciformis oocyst are underrepresented for E. tenella orthologues and can perhaps be used to understand differences between mammalian and avian Eimeria spp. oocysts. We have further identified groups of genes which are shared among apicomplexan (figure 3b and SI…) and linked them to asexual reproduction in E. falciformis. Our data indicate that genes which are characteristic for intracellular asexual reproduction stages are more conserved between E. tenella and E. falciformis, than are sporozoite defining genes. It is possible that the biological challenges in poultry and rodents faced by extracellular sporozoites are more different that intracellular conditions faced by merozoites during asexual reproduction. For considerations on drug or vaccine development further investigation into conservation status between the different poultry infecting Eimeria spp. might be of interest. In order to develop vaccines which target several species, the immune system must be activated to target conserved genes. Also in considering potential drug resistance lessons can be learned from this type of analysis, which indicates in which stage the characteristic genes are prone to change.

**Imperfect clustering might reflect true biological differences**

Oocyst and sporozoite replicates, as well as all 7 days post infection parasite samples, form separate clusters for their respective sample types (Figures 2b and 3b). The early infection pattern of no separation between day 3 and 5 post infection fits well with previous knowledge about asynchronous schizogony. In contrast, synchronization upon gametocyte formation around 7 days post infection is expected since the oocyst shedding pattern is highly reproducible. It is worthwhile to consider whether replicate separation of early infection samples reflects true biological variation. It is perceivable that the overall course and intensity of infection varies with small differences in, e.g., microbiota composition (Singer and Nash 2000; McCabe, Britton, and Parameswaran 2015), host stress levels due to cage mates, draught, differences in light exposure or other factors which do vary also in a controlled animal facility have been suggested (can we cite the editorial highlight in Science?). Variation in the course of infection triggered by such factors could explain the transcriptional profiles we see, with distinct overall patterns but replicate separation in the asexual phase. We suggest that considering such possibilities is important for interpreting results and understanding basic biology of the parasite and, in extension better understand infections in less homogeneous hosts than laboratory mice.

\*\*\*\*\*\*\* finish the below \*\*\*\*\*\*\*\*\*

Additionally, in the context of a dual RNA-seq experiment, this variation allows us to identify mRNAs which correlate in their abundance between host and parasite and go beyond the above described major patterns in the data. We thereby identified 3xxx mouse genes and x1500x parasite genes which are likely to take part in host-parasite interactions. We applied the “ISIGEM method” (Adam James Reid and Berriman 2013) to test enrichment of genes which show correlated expression pattern across host and parasite. Basically this analysis asks how likely it is that a pair of mRNAs (from 1 host gene and 1 parasite gene) are expressed simultaneously when compared to all other samples and genes in the analysis. We highlight that our reported interacting genes are not enriched in clusters reported for distinct host or parasite responses along the lifecycle (Figures 2b and 3b). Instead, they constitute an independent gene-set. Identification of these genes was possible by making use of partially unexplained variation between samples.

It is thus based on the assumption independent of the expression profile of a particular sample, host and parasite were recorded.

METHODS

*Mice and infection procedure*

Three strains of mice were used in our experiments: NMRI (Charles River Laboratories, Sulzfeld, Germany), C57BL/6 (), and *Rag1-/-* on C57BL/6 background (gift from Susanne Hartmann, FU?). Animal procedures were performed according to the German Animal Protection Laws as directed and approved by the overseeing authority Landesamt fuer Gesundheit und Soziales (Berlin, Germany). Animals where infected as described by Schmid et al., (Schmid et al. 2012), but tapwater was used instead of PBS for administration of oocysts. Briefly, NMRI mice were infected two times, which will be referred to as “first” and “second” or “challenge” infection. For the first infection, 150 sporulated oocysts were administered in 100 µL water by oral gavage. During the first infection of 60 mice, all animals were weighed every day. On day zero, before infection, as well as on 3 days post infection , 5 days post infection and 7 days post infection , caeca from 3-4 sacrificed mice per time point were collected. Epithelial cells were isolated as described in Schmid et al. (2012) to a 90% purity of epithelial cells. For challenge infection, mice recovered for four weeks before second infection. Recovery was monitored by weighing and visual inspection of fur. For the second infection, 1500 sporulated oocysts were applied by oral gavage in 100µL water. Three mice were used as non-reinfection control, referred to as day 0, second infection. *Rag1-/-* mice and the background C57BL/6 strain control mice were also subjected to first and challenge infections with 10 sporulated oocysts in 100 µL water in both cases. Samples were taken on day 0 (pre-infection control) and day 5 post infection in both first and second infections and treated as described above for NMRI mice.

*Oocyst purification for infection and sequencing*

Sporulated oocysts were purified by flotation from feces stored in potassium dichromate and administered orally in 100 uL tapwater. One E. falciformis isolate, E. falciformis Bayer Haberkorn 1970, was used for all infections and parasite samples. The strain is maintained through passage in NMRI mice in our facilities as described previously (Schmid 2012).

*Sporozoite isolation*

Sporozoites were isolated from sporocysts by in vitro excystation. For this, sporocysts were incubated at 37°C in DMEM containing 0.04% tauroglycocholate (MP Biomedicals) and 0.25% trypsin (Applichem) for 30 min. Sporozoites were purified in cellulose columns as described by Schmatz et al. (Schmatz 1997).

*RNA extraction*

Total RNA was isolated from infected epithelial cells, sporozoites, and sporulated oocysts using Trizol according to the manufacturer’s protocol (Invitrogen). Purified RNA was used to produce an mRNA library using the Illumina’s TruSeq RNA Sample Preparation guide. Sporozoites were stored in 1 mL Trizol until RNA-isolation. Total RNA was isolated using the PureLink RNA Mini Kit (Invitrogen) and reverse transcribed into cDNA. polyA selection… Simone add?

*Sequencing and quality assessment*

cDNA samples were sequenced by either GAIIX (13 samples) or Illumina Hiseq 2000 (14 samples) as specified in Table 1 (both unstranded). A fastq\_quality\_filter (FASTQ-toolkit, version 0.0.14, available at https://github.com/agordon/fastx\_toolkit.git) was applied to Illumina Hiseq 2000 samples with very lax phred score of 10. These base settings require that nine out of ten bases or more are correct in at least 60% of the bases for each read alignment. We intentionally did not use a stringent trimming before mapping to genome assemblies.

*Alignment and reference genomes*

We used the published Mus musculus mm10 assembly (Genome Reference Consortium Mouse Build 38, GCA\_000001635.2) as reference genome including annotations for mouse data. The E. falciformis genome (Heitlinger et al. 2014) was downloaded from ToxoDB (Gajria et al. 2007). For the alignment, the mouse and parasite genome files were merged into a combined reference genome, and files including mRNA sequences from both species were aligned against this reference using TopHat2, version 2.0.14, (Trapnell, Pachter, and Salzberg 2009) with –G specified, and a Bowtie2, version 1.1.2, (Langmead and Salzberg 2012). This was done to avoid spurious mapping in ultra-conserved genomic regions. Single-end and pair-end sequence samples were aligned separately with library type 'fr-unstranded' specified for pair-end samples. Bam files (Frazee et al. 2015) were used as input for the function “featureCounts” from of the R package “Rsubread” (Liao, Smyth, and Shi 2014).

*Differential mRNA abundance, data normalization and sample exclusions*

After import of data to R, mouse and parasite data was separated using obvious patterns in transcript IDs and onwards analyzed separately. For each species separately, count data was normalized using the R-package edgeR (version 3.14.0; cite) with the upperquartile normalization method. Briefly, genes with a coverage below 3000 (mouse ) and XXX (E.falciformis) summed over all samples (libraries) were removed and normalization factors were calculated for the 75% quantile for each library. This normalization is suitable for read densities following a negative binomial distribution. We excluded samples NMRI\_2nd\_3days post infection \_rep1 and NMRI\_2nd\_5days post infection \_rep2 due to low parasite contribution (0.012% and 0.023%) to the overall transcriptome. Technically, this exclusion made it possible to obtain parasite read counts in agreement with a negative binomial distribution (see additional file x). It is likely that the number of reads in the excluded samples would have been insufficient to fully normalize these datasets to those with the highest parasite contributions. Both excluded samples are from challenge infection and it is likely that the infected mice were immune to re-infection. One additional sample (NMRI\_1stInf\_0days post infection \_rep1) was excluded because the uninfected control showed unexpected mapping of reads to the E. falciformis genome (0.033%). We used EdgeR to fit generalized linear models to the kept samples (GLMs with a negative binomial link function) for each gene (glmFit) and to perform likelihood ratio tests for models with or without a focal factor (glmLRT). As samples and individual replicates were sequenced in batches to different depth and using different instrumentation (Table 1) we performed multidimensional scaling of samples as quality controls (additional files xyz). These confirm the absence of batch effects influencing analysis and quality of results.

*Selection of differentially abundant mRNAs and hierarchical clustering*

Mouse mRNAs responding to infection or differently abundant at different timepoints of infection (0 vs “any days post infection ” or “any days post infection ” vs “any days post infection ”; see Table 2) and E. falciformis genes showing differences between any life cycle stage (oocysts vs sporozoites, or any of those vs. “any days post infection ” or “any days post infection ” vs. “any days post infection ”) were selected and used for hierarchical clustering. Hierarchical clustering was performed using the complete linkage method based on Euclidean distances.

*Enrichment tests*

Gene Ontology (GO) enrichment analysis was performed using the R-package topGO with the “weight01” algorithm and Fisher's exact tests. We additionally perfomrmed a correction for multiple testing on the returned p-values (p.adjust using the BH-method). Similarly a Fisher's exact test and corrections for multiple testing were also used to test for an overrepresentation of transcripts with a signal sequence for entering the secretory pathway or with transmembran domains (as inferred using Signal P).

Evolutionary conservation of gene families was analyzed based from categories from Heitlinger et al. (2014) which are as follows: i) E. falciformis specific, ii) specific to the genus Eimeria, compiled by an analysis of E. falciformis, E. maxima and E. tenella, iii) Coccidia: Eimeria plus T. gondii and Neospora caninum, iv) Coccidia plus Babesia microti, Theileria annulata, Plasmodium falciparum and Plasmodium vivax v) the same apicomplexan parasites plus Cryprosporidium hominis, vi) universally conserved in the eukaryote super-kingdom inferred from an analysis of Saccharomyces cerevisiae and Arabidopsis thaliana. These categories were tested for overrrepresentation in gene-sets with particular patterns described in the text using Fisher's exact-tests and the resulting p-values were again corrected for multiple testing.

*Correlation analysis of apicomplexan transcriptomes*

Transcriptome datasets from Reid et al (2014), Walker et al. (2014) and Hehl et al (2012) were downloaded from ToxoDB (ref). Orthologues between E. falciformis, E. tenella and T. gondii were compiled as defined in Heitlinger et al. (2014) and only 1:1:1 otholog triplets were retained for analysis, as multi-paralog gene-families might contain member showing divergent evolution of gene-expression due to neo/sub functionalisation. Spearman correlation coefficients for expression over different samples in all studies and over different species represented by their orthologues were determined. Hierarchical clustering with complete linkage was used to cluster resulting correlations coefficients.

*Correlation analysis between host and parasite*

We used the ISIGEM method of Reid and Berriman (2013) to asses correlations of host and parasite transcripts. This method avoids spurious correlations for host-parasite gene pairs remaining e.g. unchanged over many conditions. It does so by estimating emmpircal p-values (ISIGEM-scores), which correspond to the proportion of cases in which a random shuffeling of samples produces better correlations than those observed in the given samples between host-parasite pairs. Similar to Reid and Berriman (2013) we used 5x10⁵ replicates of random shuffels and did not correct for multiple testing in our set of (79,764,454 tested correlations between 13,343 mouse and 5978 parasite genes).

All analyses were performed in R (cite R-core). Complete scripts are available at [https://github.com/derele/Ef\_RNAseq.git tagged as version 1.0](https://github.com/derele/Ef_RNAseq.git%20tagged%20as%20version%201.0).

*Orthologue assignments*E. falciformis genes which were analyzed in detail and orthologues identified by manually entering E. falciformis gene names in ToxoDBs “Gene\_ID” search.

**COMPETING INTERESTS**

The authors declare that they have no competing interests.

**AUTHOR CONTRIBUTIONS**

Animal and parasite experiments: Simone Spork, experimental design: Richard Lucius, Simone Spork, Emanuel Heitlinger, RNA sequecing: Christoph Dieterich, data anylsis: Emanuel Heitlinger, Totta Kasemo, text: all?

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**Tables**

Table 1: Summary of data sorted per sample. Sequencing method, experimental batch, total reads sequenced, reads mapping to mouse genome, reads mapping to *E. falciformis* genome, percentage of *E. falciformis* reads, and number of *E. falciformis* genes with detected transcripts are shown.

\* Sample names are given with information separated by underscore as follows: 1) mouse strain, 2) first or second infection, 3) day post infection (days post infection ), and 4) replicate number.  
\*\* Percentage of *E. falciformis* reads is provided as percentage of total (host plus parasite) reads.

**Figures** (Legends only; figures in separate files)

Figure 1. Oocyst output and changes in intensity of E. falciformis infection in mouse. A) Oocyst counts in first and challenge infection are shown for different mouse strains. Immune competent mice (NMRI and C57BL/6) have reduced oocyst counts in challenge infection, whereas immune incompetent Rag1-/- mice do not. B) RT-qPCR data of E. falciformis 18S in NMRI mice displays an increase in parasite material over the course of infection. Less parasite material is detected in challenge infected mice. C) The percentage of parasite mRNA detected by RNA-seq increases during infection. More material is detected in naïve mice compared to challenge infected mice, as is also demonstrated in B. Sporozoites and oocysts are shown with 100% parasite material.

Figure 2. Differential abundance of mouse mRNAs and clutering of abundance profiles – A) Log2 fold-changes obtained from a comparison of RNAseq data at 7days post infection vs. unifected controls are plotted against log2 fold changes obtained in microarray study (Schmid et al. 2014) at 6days post infection vs. similar controls. B) I) Overlap between genes showing differential abundance (FDR < 0.01; EdgeR glm likelihood-ratio tests) between uninfected controls and different time-points after infection and ii) between different time points and all those genes reacting to infection is viszalized in venn diagramms (C) Hierarchical clustering of expression profiles for these mRNAs performed on Euklidean distances using complete linkage. Cluster cut-offs were set to identify gene-sets with profile interpretable in relation to the parasite lifecycle and between mice of different immune competence.

Figure 3. Differential abundance of E. falciformis mRNAs and clutering of abundance profiles – A) i) Overlap between genes showing differential abundance (FDR < 0.01; EdgeR glm likelihood-ratio tests) between intracellular stages at 3days post infection , 5days post infection and 7days post infection . ii) Overlapp between earls vs. late chanes deduced from this and between “environmental stages” (oocysts and sporozoites) (C) Hierarchical clustering of expression profiles for these differentially abundand mRNAs performed on Euklidean distances using complete linkage. Cluster cut-offs were set to identify gene-sets with profile interpretable in relation to the parasite lifecycle.

Figure 4: Correlation of the E. falciformis transcriptome with other coccidian transcriptome- Correlations of our mRNA abundance data with orthologues from published RNA-seq transcriptomes from E. tenella (Reid et al. 2014, Walker et al. 2014) and T. gondii (Hehl et al 2012) are shown. Correlations coffeicients (Spearman's rho) were clustered using complete linkage. T. gondii and Eimeria spp. “late infection” samples cluster together. E. falciformis early infection samples cluster with E. tenella merozoites. E. falciformis sporozoites cluster with E. falciformis early infection, whereas oocysts cluster with E. tenella unsporulated oocysts.

Figure 5: Pairwise interaction of host and parasite genes deduced from correlated expressions – TO BE ADDED

**ADDITIONAL FILES**

**Additional file 1**: Raw and normalized counts

Raw counts of reads mappins to the E. falciformis and mouse genome for individual samples in our study. Normalized counts for

separately for the host and parasite mappings (three compressed csv files).

**Additional file 2:** Results of statistical tests (edgeR)

Focal contrast, fold-changes, likelihood ratio in/excluding this

difference in models, p-values , and false discovery rates (adjusted p-values) are given for all tested contrasts (one compressed csv file).

**Additional file 3:** Additional methods and results

Document containing additional figures and summary tables (pdf).

**Additional file 4**: Results of enrichment analyses (topGO)

**Additional file 5**: Results of enrichment analysis for categories of differently conserved gene orthologues

**Additional file 6**: Result of metabolic pathway enrichment of sporozoite defining genes

Tables listing all tested gene sets and resulting significant GO terms.

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