

Hammondia hammondi, an avirulent relative of Toxoplasma gondii, has functional orthologs of known T. gondii virulence genes

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Toxoplasma gondii is a ubiquitous protozoan parasite capable of infecting all warm-blooded animals, including humans. Its closest extant relative, *Hammondia hammondi*, has never been found to infect humans and, in contrast to *T. gondii*, is highly attenuated in mice. To better understand the genetic bases for these phenotypic differences, we sequenced the genome of a *H. hammondi* isolate (HhCatGer041) and found the genomic synteny between *H. hammondi* and *T. gondii* to be >95%. We used this genome to determine the *H. hammondi* primary sequence of two major *T. gondii* mouse virulence genes, *TgROP5* and *TgROP18*. When we expressed these genes in *T. gondii*, we found that *H. hammondi* orthologs of *TgROP5* and *TgROP18* were functional. Similar to *T. gondii*, the *HhROP5* locus is expanded, and two distinct *HhROP5* paralogs increased the virulence of a *T. gondii* *TgROP5* knockout strain. We also identified a 107 base pair promoter region, absent only in type III *TgROP18*, which is necessary for *TgROP18* expression. This result indicates that the *ROP18* promoter was active in the most recent common ancestor of these two species and that it was subsequently inactivated in progenitors of the type III lineage. Overall, these data suggest that the virulence differences between these species are not solely due to the functionality of these key virulence factors. This study provides evidence that other mechanisms, such as differences in gene expression or the lack of currently uncharacterized virulence factors, may underlie the phenotypic differences between these species.

comparative genomics | gene content versus gene deployment | pathogenesis | host range

Approximately 20% of the US population is infected with the intracellular parasite *Toxoplasma gondii* (1). *T. gondii* is a member of the phylum Apicomplexa, which includes multiple human and animal pathogens, including the causative agents of malaria and cryptosporidiosis. A unique feature of this parasite compared with its apicomplexan relatives is the ability to infect, and cause disease in, nearly all warm-blooded animals studied to date, including birds (2). *T. gondii* is primarily asymptomatic in healthy humans but can cause severe disease in utero, in the immunocompromised, and in healthy adults (3–5). In the laboratory, the mouse model of toxoplasmosis has been extensively studied, a relevant model given that rodents are hosts for *T. gondii* in both rural and urban environments (6, 7). Highly virulent infections with *T. gondii* can be initiated by inoculation with fast-growing tachyzoites, slow-growing bradyzoite tissue stages (8), and oocysts (2). Felines are the definitive (i.e., sexual) host for *T. gondii* (9), and rodent to cat transmission via predation can expand the parasite population by up to 10 million-fold.

The closest extant relative of *T. gondii* is *Hammondia hammondi* (10, 11), a fellow apicomplexan that also has its sexual cycle in cats (10). This parasite was once thought to be a strain of *T. gondii* (12), but it is now accepted as a distinct species (2). Experimentally, a variety of intermediate hosts have been successfully

infected with *H. hammondi* (including rabbits, pigs, rodents, and monkeys), but birds are refractory to infection (ref. 13, and reviewed in ref. 2). In contrast to *T. gondii*, in both wild-type and IFN- γ knockout mice, parenteral *H. hammondi* infections with all parasite life stages are nonlethal (2), although oral infections with high numbers of *H. hammondi* oocysts of some strains can cause disease (2) and mortality (13). In infected mice, *H. hammondi* tachyzoites and cysts can be found in several tissues, including lymph nodes and spleen (2). Importantly, *H. hammondi* tissue cysts (bradyzoites) from intermediate hosts are not infective to mice by any route, and therefore horizontal transmission between intermediate hosts is a unique feature of *T. gondii* (2, 13).

Why some humans become sick from *T. gondii* infection whereas most remain asymptomatic is largely unknown. Recently, attention has been focused on the genetic basis of parasite virulence, and mice have been used as a model for virulence in *T. gondii*. Among the virulence determinants, the most potent are rhoptry proteins 18 and 5 (*TgROP18* and *TgROP5*), both members of a large superfamily of proteins secreted from parasite organelles called rhoptries (14–17). Subsequent work has shown that *TgROP18* directly phosphorylates immune-related GTPases (IRGs), which prevents them from being efficiently loaded onto, and disrupting, the parasitophorous vacuole (PV) (18–24). For *TgROP5*, complete knockout of this locus in the hypervirulent type I strain renders the parasite completely avirulent in mice (15, 16), and this phenotype is due, at least in part, to *ROP5* binding directly to IRG proteins, which presumably then permits *TgROP18* IRG phosphorylation and inactivation (23, 24). Based on recent sequence analysis of the *Neospora caninum* genome [a parasite structurally similar to *T. gondii* but with a sexual cycle in dogs (25, 26)], it has been suggested that the orthologs of *TgROP18* and *TgROP5* are not functional as virulence genes in this species. Specifically, *ROP18* is a pseudogene in *N. caninum* (25), and the *NcROP5* locus has not significantly expanded in this species as it has in *T. gondii*, having only 2 copies compared with 4–10 in *T. gondii* (15, 16, 25). In addition, the *N. caninum* *ROP18* ortholog has a similar promoter structure as the “avirulent” *TgROP18* allele in *T. gondii* strains with low *TgROP18* transcript abundance (27).

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The authors declare no conflict of interest.

Data deposition: The HhCatGer041 genomic assembly and raw sequence reads have been deposited in GenBank under BioProject PRJNA191036 and BioSample 1974510.

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The genetic bases for the dramatic differences in mouse virulence between *T. gondii* and *H. Hammondii* are unknown. The goal of the present study was to sequence the genome of *H. Hammondii* strain HhCatGer041 and use it to determine the overall degree of genomic identity and synteny between HhCatGer041 and *T. gondii*. We then functionally characterized the *H. Hammondii* orthologs of TgROP18 and TgROP5 (HhROP18 and HhROP5) to determine whether they could be linked, or not, to the phenotypic differences between these closely related species.

Results

***H. Hammondii* Genome Has High Synteny and Similarity to That of *T. gondii*.** Based on previously published analyses of internal transcribed spacer sequences (11, 28), *H. Hammondii* has been categorized as the nearest extant relative of *T. gondii*, and our rough draft of the *H. Hammondii* genome confirms this similarity. Details about the sequence reads and assembly are in Tables S1 and S2. The genome assembled into 56,472 scaffolds, with the largest being 81 kb. When reads were mapped to the v7.2 draft of the *T. gondii* genome (strain GT1; www.toxodb.org), average coverage for nucleotides with >3× coverage was ~20×, and 68% of the GT1 nucleotides had ≥4× coverage (Fig. S1). Based on these alignments, the overall divergence genome-wide is 4.9%. The largest region without any sequence coverage was found on the first 175 kb of *T. gondii* Chr IX (Fig. S1). Despite its incomplete nature, the assembly provides strong evidence for a high degree of genomic synteny between these species. Specifically, strict reciprocal BLAST analysis of all predicted *T. gondii* coding regions (including introns) on 87 contigs >20 kb in length shows that 298 of 311 genes (~96%) are in the same order and orientation as they are in *T. gondii*. Importantly, only 96 genes (out of 8,993 queried) had no significant reciprocal blast hits ($E < 1e^{-5}$; Dataset S1). An identical comparison with *N. caninum* genes shows that 818 out of 7,226 predicted genes have no reciprocal blast hits [although more robust estimates of shared gene content between *T. gondii* and *N. caninum* have been published by others (25)]. Whereas the number of genes “missing” in *H. Hammondii* compared with *T. gondii* will change as better assemblies of the *H. Hammondii* genome emerge, this rough draft further establishes *H. Hammondii* as the closest extant relative of *T. gondii* and sets the stage for gene-by-gene comparisons to better understand the evolution of virulence in *T. gondii*.

ROP5 Locus Is Expanded, Diversified, and Functionally Conserved In *H. Hammondii*. Recently we and others demonstrated a clear role for the TgROP5 locus in *T. gondii* virulence (15, 16). This locus was characterized by the presence of multiple paralogs that show clear evidence for selection-driven diversification (15). Using the *H. Hammondii* genome sequence, we examined sequence read coverage on scaffolds with *H. Hammondii* ROP5 sequence (Fig. 1A), and this analysis suggested that the ROP5 locus is also expanded in *H. Hammondii*, with an estimated copy number of 8–10. To determine whether the locus had also diversified, we PCR-amplified and sequenced eight HhROP5 paralogs and identified eight distinct protein coding sequences (Fig. 1B, Fig. S24), confirming that this locus is expanded and diversified in *H. Hammondii* as it is in *T. gondii* (all primers used in this study are listed in Table S3). This finding is in contrast to what has been reported in *N. caninum* (25) although the role of NcROP5 in mouse virulence has not been directly tested. All eight isoforms harbored the same sequence features and domains as TgROP5 orthologs, including predicted signal sequences, an amphipathic helical domain in the N terminus that mediates interaction with the PV in TgROP5 (29), and a clear pseudokinase domain (Fig. S24). Selection analyses also revealed that these individual copies showed strong evidence for selection. For example, isoform 2–4 has a non-synonymous/synonymous substitution ratio (dN/dS) of >2.8 compared with isoforms 1–3, 1–4, and 1–5, suggesting

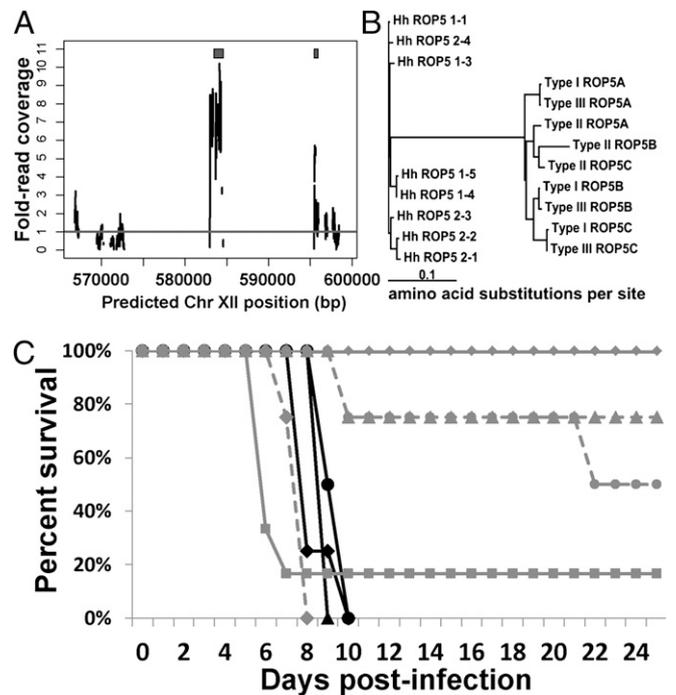


Fig. 1. Expansion, diversification, and functionality of *H. Hammondii* ROP5. (A) Sequence coverage plot for three *H. Hammondii* genomic scaffolds arranged based on their putative location on *H. Hammondii* Chr XII (determined by Nucmer alignment of *H. Hammondii* scaffolds to the v7.2 GT1 genome). Black blocks above the plots indicate the location of significant (expect $< 1 \times 10^{-10}$) TBLASTN hits of TgROP5B against the *H. Hammondii* genome. (B) Phylogram of amino acid sequences of eight HhROP5 paralogs and known TgROP5 paralogs. (C) Mice were infected with RH:WT (black lines) or RH Δ ROP5 complemented with a single copy of either HhROP5₁₋₁ (gray solid lines) or HhROP5₂₋₁ (gray dashed lines). Four mice were used for each strain and dose with the exception of RH Δ ROP5:HhROP5₁₋₁ at 10^6 ($n = 6$), and RH:WT at 10^4 ($n = 3$). Tachyzoite dose symbol key: ■, 10^6 ; ◆, 10^5 ; ▲, 10^4 ; ●, 10^3 .

not only that the *HhROP5* locus has expanded in *H. Hammondii* but also that it has been under selection.

Based on our sequence analyses of *HhROP5*, we chose two paralogs, 1–1 and 2–1 (Fig. 1B, Fig. S24), for complementation into a type I *T. gondii* strain lacking the entire TgROP5 locus [RH Δ TgROP5 (15)]. In contrast to the parental strain (RH), which has an effective LD₁₀₀ of a single parasite (30), this strain does not cause mortality at doses as high as 10^6 tachyzoites (15, 16). When expressed in *T. gondii*, HhROP5 paralogs 1–1 and 2–1 are both effectively trafficked to the *T. gondii* rhoptries (Fig. S3B) and can be found associated with the PV (Fig. S3E) as expected. Fusing 713 and 702 bp of upstream sequence from HhROP5₁₋₁ and HhROP5₂₋₁, respectively (Figs. S2B and S3A), with luciferase is capable of driving reporter expression at levels similar to one another and to a homologous upstream sequence for *T. gondii* type II ROP5B (Fig. S3A). In multiple experiments, it was found that the promoter activity of HhROP5₂₋₁ is slightly lower than HhROP5₁₋₁. Anti-HA Western blotting on parasites expressing these paralogs is consistent with this slight difference in promoter activity: normalized protein levels for HhROP5₂₋₁ are slightly lower than for HhROP5₁₋₁ (Fig. S3C and D). To determine whether these HhROP5 orthologs were functionally conserved as virulence genes in *H. Hammondii*, we infected mice with two genetically distinct clone sets of RH Δ ROP5:ROP5Hh₁₋₁ and RH Δ ROP5:ROP5Hh₂₋₁, along with RH Δ ROP5 and wild-type RH (Fig. S44). For clone set 1, RH Δ ROP5:ROP5Hh₂₋₁ was 100% lethal at a dose of 10^5 , and 50% lethal at a dose of 10^3

infection and showed comparatively low levels of parasite burden over the course of the infection (Fig. 3 A–C). All mice infected with III:TgROP18_I and III:TgROP18_{II} died by day 16 (Fig. 3B). Remarkably, >80% of mice infected with III:HhROP18 died before mice infected with any of the other strains, including those infected with III:TgROP18_I (Fig. 3B). This difference in time to mortality is consistent with the in vivo BLI data. Specifically, III:HhROP18 increased in number in infected mice much more rapidly than all other strains, being over 10-fold higher than all other strains on days 6 and 7 postinfection (Fig. 3A). These data show that, despite being derived from a parasite species that is attenuated in mice, the *H. hammondi* ROP18 gene, when expressed in

T. gondii, may actually be a more effective virulence gene than the alleles from *T. gondii* types I and II. Based on quantitative Western blotting, HhROP18 is expressed at similar levels as TgROP18_I (Fig. S5 B and C), suggesting that the *HhROP18* allele, rather than differences in expression level, are responsible for the increased impact of *HhROP18* on virulence compared with the *T. gondii* ROP18 alleles from type I and type II strains.

Discussion

The differences in virulence between *H. hammondi* and *T. gondii* are belied by their close phylogenetic relationship. In contrast to *T. gondii*, when oocysts are injected subcutaneously or

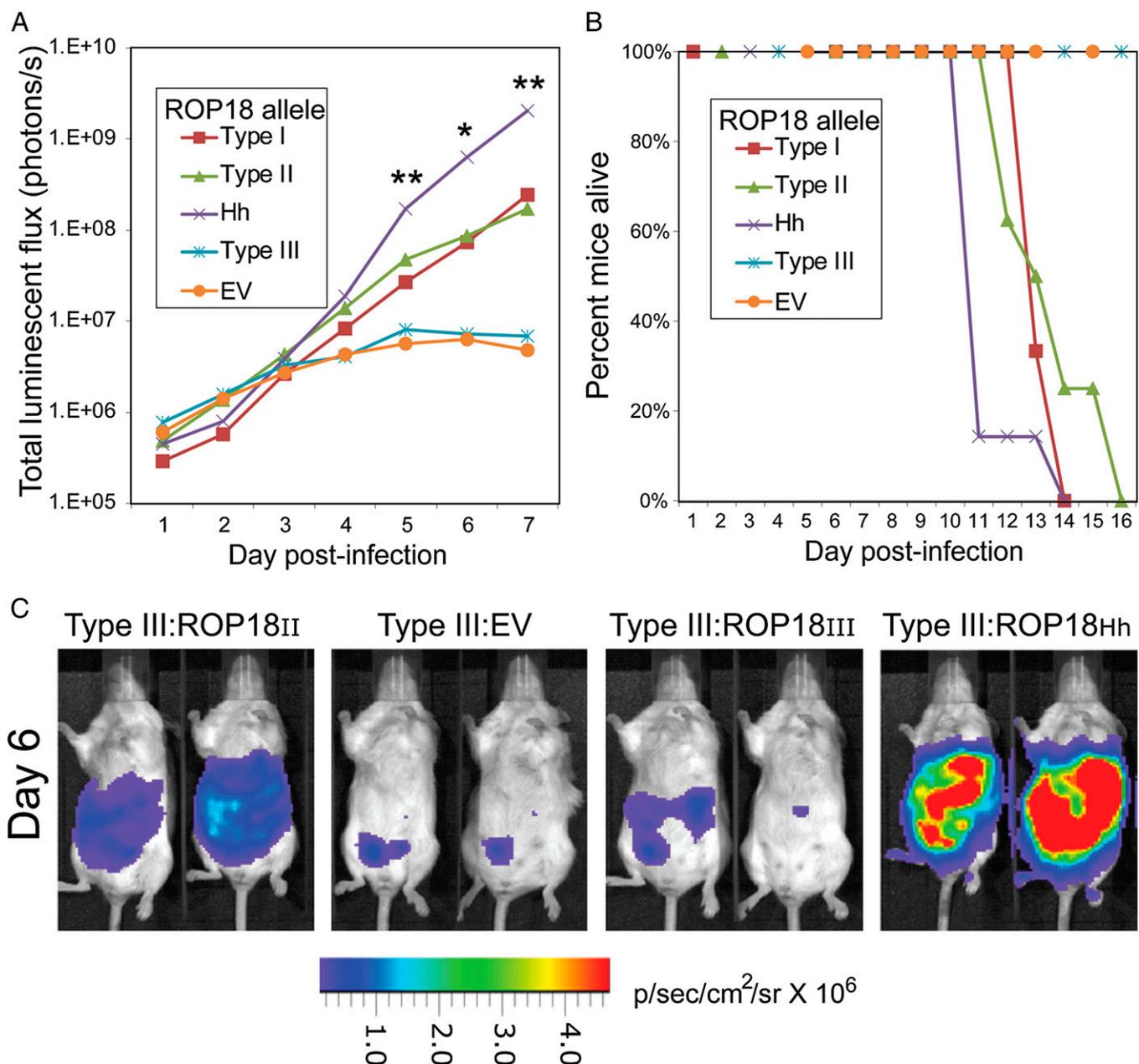


Fig. 3. Expressing HhROP18 in a TgType III background increases parasite lethality and in vivo proliferation. Mice were infected with 10³ parasites of a type III strain complemented with TgROP18_I, TgROP18_{II}, TgROP18_{III}, HhROP18, or empty vector (*n* = 3, 8, 5, 7, and 5, respectively). Results shown are pooled from two experiments. (A) Quantitative in vivo BLI of infected mice. Asterisks indicate significantly higher luciferase signal in HhROP18-complemented parasites compared with TgROP18_I-complemented parasites (**P* < 0.05; ***P* < 0.01). (B) Mouse mortality. (C) Representative images of parasite burden in mice on day 6 postinfection.

intraperitoneally, *H. hammondi* is completely avirulent in wild-type and IFN- γ knockout mice (2) and has never been found to infect humans. A number of mechanisms could explain the dramatic virulence differences between two species that share such a recent common ancestry. One is that virulence is driven by genes that are uniquely present and/or divergent in *T. gondii* compared with *H. hammondi*. Another is that both species harbor the same arsenal of effectors, but they are differentially regulated by distinct transcriptional mechanisms or transcription factors. However, another is that the *H. hammondi* genome contains “avirulence” genes that are only present and/or expressed in *H. hammondi*. Genome sequence comparison between these organisms is a crucial first step in addressing these hypotheses and to ultimately determine how mouse virulence, as well as other traits, evolved uniquely in *T. gondii*.

In the present study, we found that *H. hammondi* orthologs of *ROP5* and *ROP18*, which are the most potent mouse virulence factors known in *T. gondii* (14–17), are functional when expressed heterologously in *T. gondii*. As in *T. gondii*, the *HhROP5* locus has expanded, and individual paralogs appear to be under diversifying selection in *H. hammondi*. Specifically, *H. hammondi* *ROP5*₂₋₁ increases the virulence of a *T. gondii* *ROP5* knockout strain 100-fold, and this effect is more potent than complementation of the same *ROP5* knockout strain with a single copy of *T. gondii* *ROP5* [the *ROP5* “A” isoform (15)]. Whereas $\text{RH}\Delta\text{TgROP5}:\text{TgROP5A}$ was lethal to only 20% at a dose of 10^5 tachyzoites, we observed 100% mortality with this dose of $\text{RH}\Delta\text{TgROP5}:\text{ROP5Hh}_{2-1}$, and 50% mortality at a dose of 1,000 $\text{RH}\Delta\text{TgROP5}:\text{Hh}_{2-1}$ tachyzoites. Interestingly, in terms of mouse mortality, *HhROP5*₁₋₁ was less potent than *HhROP5*₂₋₁. Overall these data indicate that the *ROP5* locus in *H. hammondi* bears all of the hallmarks of the orthologous locus from *T. gondii*: (i) it contains multiple copies that have undergone selection-driven diversification in sequence, (ii) individual copies can increase the pathogenesis of a *T. gondii* *ROP5* knockout, and (iii) individual copies vary in terms of their effects on virulence. Interestingly, across the *H. hammondi* *ROP5* paralogs, the most polymorphic region is found within the pseudokinase domain in residues 490–501, and a similar level of polymorphism is found in this same region across the *T. gondii* *ROP5* paralogs (Fig. S24) (15). Future work on the other six *H. hammondi* *ROP5* paralogs will provide more information on the impact of locus diversification in this species. Moreover, we have not yet tested the impact of expressing multiple distinct copies of *H. hammondi* *ROP5* orthologs, nor the impact of *H. hammondi* *ROP5* expression on the loading of IRG GTPases (23, 24). These studies should help to inform current models of TgROP5 structure-activity relationships in *T. gondii*.

In *T. gondii*, virulence gene expression, rather than individual amino acid polymorphisms, has been linked to differences in virulence between strains. Specifically, *TgROP18* transcript abundance in type III strains is significantly lower than in members of the type I and II lineages, and, within the *T. gondii* species, this lower transcript abundance is clearly due to a 2.1-kb sequence inserted 85 bp upstream of the *ROP18* start codon compared to strain types I and II (27, 31). We found that a similarly homologous insertion was present in the *H. hammondi* *ROP18* upstream sequence. Given the presence of this insertion, we were surprised to find that, in contrast to the corresponding sequence from *T. gondii* type III strains, the *H. hammondi* *ROP18* promoter drove significantly high levels of luciferase in promoter assays conducted in *T. gondii*. We identified a 107-bp region in *H. hammondi* and type I and II *T. gondii* *ROP18* upstream of the predicted transcriptional start site that is not present in *T. gondii* type III *ROP18*. Deletion of this sequence in *H. hammondi* *ROP18* destroys promoter activity, suggesting that this sequence contains a core *ROP18* promoter or enhancer element in both species (at least when heterologously expressed in *T. gondii*). Even more exciting, inserting this *H. hammondi* sequence into the comparatively

ineffective type III promoter at the proper location effectively resurrects this promoter to levels that are even higher than the *TgROP18*_{II} promoter. Importantly, based on BLASTN, this 107-bp sequence cannot be identified in the entire *N. caninum* genome (expect >10), suggesting that the *ROP18* promoter became “active” after the split between the *N. caninum* and *T. gondii*/*H. hammondi* lineages. This increased promoter activity has significant phenotypic consequence: *HhROP18* appears to be at least as potent as, and possibly MORE potent than, *TgROP18*_I.

Through a preliminary sequence analysis of an isolate of *H. hammondi* (*HhCatGer041*), we have found that orthologs of key *T. gondii* mouse virulence genes are functionally conserved in this species. Whereas virulence commonly evolves in pathogenic bacteria by changes in gene content via acquisition of so-called pathogenicity islands [e.g., in pathogenic *Escherichia coli* (32)], in the case of the eukaryotic pathogens *T. gondii* and *H. hammondi*, at least, gene content and functionality may not be sufficient to explain their phenotypic differences. One hypothesis is that the inability of *H. hammondi* to infect and be transmitted by mice compared with *T. gondii* is due to differences in gene deployment. This explanation for the phenotypic differences between *T. gondii* and *H. hammondi* would be somewhat analogous to what has been found in transcriptome comparisons between free-living and “parasitic” forms of *Listeria monocytogenes* where highly distinct transcriptional profiles, driven by activation/deactivation of key transcription factors, are the hallmark of the pathogenic lifestyle (33). High quality data from the *H. hammondi* transcriptome will be necessary to address this hypothesis. Transcriptome analyses in *H. hammondi* are made more difficult by the fact that *H. hammondi* does not replicate for more than 5 d in cell culture and therefore must be generated directly from cat-derived oocyst stages. However, the depth of next generation sequencing provides a potential solution to this problem. These analyses will be facilitated by the crude *H. hammondi* genome draft presented here as well as the recently released draft of a different isolate (H.H.34) by the J. Craig Venter Institute (<http://jvci.org>).

Methods

Parasite Strains and Maintenance. *T. gondii* parasites were allowed to invade monolayers of human foreskin fibroblasts (HFFs) that were grown at 37 °C in 5% CO₂. HFFs were maintained in Dulbecco’s modified Eagle’s medium with 10% (vol/vol) FCS, 2 mM glutamine, and 50 $\mu\text{g}/\text{mL}$ each of penicillin and streptomycin (cDMEM). RH, ME49, and CEP were used as representative type I, II, and III strains, respectively. The *H. hammondi* strain (*HhCatGer041*) was isolated from cat feces on June 29, 2009 during a survey of *Toxoplasma gondii* in Germany. The isolate was confirmed as *Hammondia hammondi* as previously described (34) using specific *Hammondia hammondi* primers (35) and by the complete nonvirulence of the parasite in IFN- γ knockout mice. For *H. hammondi* oocyst production, IFN- γ knockout mice were fed 10^4 *H. hammondi* oocysts and killed 59 d later. Muscles from infected mice were fed to 10- to 20-wk-old cats, and feces were collected during days 5–11 postinfection. Unsporulated oocysts were isolated by sucrose floatation and allowed to sporulate at room temperature in 2% sulfuric acid (2).

DNA Isolation from *H. hammondi* Oocysts. Sporulated oocysts (40–80 million) were washed four times in Hanks’ balanced salt solution (HBSS) and treated with 10% bleach in PBS for 30 min. Washed pellets were resuspended in 4 mL of HBSS in a 15-mL falcon tube, and 1 g of sterile glass beads (710–1,180 μm ; Sigma) were added. Parasites were vortexed on high speed for 30 s, cooled for 30 s, and then vortexed for 30 s more. The supernatant was removed and pelleted by centrifugation. DNA was isolated from this preparation, which contained sporocysts that had been freed from the oocyst using the DNAzol reagent according to the manufacturer (Life Technologies). For two different preparations, 8 ng of DNA was linearly amplified using the GenomiPhi DNA Amplification Kit (GE Healthcare Life Sciences) and ethanol precipitated. These preparations were used for Illumina and PacBio RS (Pacific Biosciences) library preparation and sequencing and for PCR amplification (described in *Gene Identification, Sequencing, and Generation of Transgenic T. gondii*).

Sequencing, de Novo Assembly, and Analysis of the *H. hammondi* Genome. For Illumina sequencing, two different preparations of linearly amplified

H. hammondi DNA were combined, and a single library (insert size ~200 bp) was made from the preparation using the DNA TrueSeq kit. Thirty million 36-bp paired-end reads were generated in a single lane of an Illumina Genome Analyzer Ix at the Tufts Genomics Core Facility. Mean insert size and SD were calculated via de novo assembly in Ray (36). Two additional linearly amplified DNA samples were combined and submitted to the Duke Genomics Core Facility, and sequence data from four single molecule, real-time (SMRT) cells were obtained from a 2-kb insert library on a PacBio RS system. Consensus PacBio RS reads were assembled along with the Illumina reads using Ray with the following settings: K-mer size of 21, a mean insert size of 206, and a SD of 33. The K-mer size was determined iteratively by running the assembly using K-mer sizes ranging from 12 to 23 to maximize the N50 value. Synteny analyses were performed by reciprocal BLASTN between the genomic DNA coding regions for all v7.2 ME49 predicted genes versus the *H. hammondi* genomic contigs with an expected value of $1e^{-5}$. Genes on contigs larger than 20 kb were then scored manually for reciprocal hit presence, order, and orientation. Raw Illumina sequence reads were aligned to the v7.2 GT1 *T. gondii* genome using Bowtie2 (<http://bowtie-bio.sourceforge.net/bowtie2/index.shtml>) using the following parameters: bowtie2 -local -L 10 -k 10 -i S,1,0.5. Coverage plots were generated using Samtools mpileup on the bowtie2 output, and coverage was determined at each base using a custom perl script.

Gene Identification, Sequencing, and Generation of Transgenic *T. gondii*.

H. hammondi contigs with *ROP5* and *ROP18* coding and upstream sequences were identified using TBLASTN with *T. gondii* ROP5 and ROP18 protein sequences. HhROP5 isoforms were PCR amplified using short-cycle, long-

extension PCR to amplify ~1,000 bp upstream of the predicted start codon to the predicted stop codon (Invitrogen). Eight distinct clones were sequenced and protein sequences aligned using CLUSTALW (37). Two paralogs (1-1 and 2-1) were amplified directly from the cloning plasmid (PCR2.1 TOPO) using primers with Ascl (Forward) and XmaI (Reverse) sites and cloned into the pUPRT-HA vector (15) in frame with an HA tag. Selection for plasmid integration into RHΔ*TgROP5* was performed using 5-fluorodeoxyuridine (38), and resistant parasites were cloned by limiting dilution. Sequences for *Hh*, *Tg_I*, *Tg_{II}*, and *Tg_{III}* *ROP18* were directionally cloned into pENTR-D-Topo (Invitrogen) and recombined into a derivative of the pGRA-HA-HPT vector (14) in which the *Gra1* upstream sequence was replaced with the *attR1-ccdB-attR2* cassette for Gateway recombination cloning (Invitrogen) (39, 40). The constructs were transfected into a click beetle luciferase-expressing *T. gondii* strain null for HXGPRT (CEP) (14). Parasites were selected for resistance to mycophenolic acid and xanthine and cloned by limiting dilution (41). PCR was used to verify the presence of the full construct in III:*TgROP18_{III}* parasites. All clones were validated using anti-HA immunofluorescence and Western blotting. Primers are listed in Table S3.

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