



Published in final edited form as:

Science. 2012 January 13; 335(6065): 218–221. doi:10.1126/science.1210829.

A DOC2 Protein Identified by Mutational Profiling is Essential for Apicomplexan Parasite Exocytosis

Andrew Farrell^{1,*}, Sivasakthivel Thirugnanam^{1,*}, Alexander Lorestani^{1,*}, Jeffrey D. Dvorin^{2,3,*}, Keith P. Eidell¹, David J.P. Ferguson⁴, Brooke R. Anderson-White¹, Manoj T. Duraisingh^{2,†}, Gabor T. Marth^{1,†}, and Marc-Jan Gubbels^{1,†}

¹Department of Biology, Boston College, Chestnut Hill, MA 02467, USA

²Department of Immunology and Infectious Diseases, Harvard School of Public Health, Boston, MA 02115, USA

³Division of Infectious Diseases, Children's Hospital Boston, Boston, MA 02115, USA

⁴Nuffield Department of Clinical Laboratory Science, University of Oxford, John Radcliffe Hospital, Oxford OX3 9DU, UK

Abstract

Exocytosis is essential to the lytic cycle of apicomplexan parasites and required for the pathogenesis of toxoplasmosis and malaria. DOC2 proteins recruit the membrane fusion machinery required for exocytosis in a Ca²⁺-dependent fashion. Here, the phenotype of a *Toxoplasma gondii* conditional mutant impaired in host cell invasion and egress was pinpointed to a defect in secretion of the micronemes, an apicomplexan-specific organelle that contains adhesion proteins. Whole genome sequencing identified the etiological point mutation in TgDOC2.1. A conditional allele of the orthologous gene engineered into *Plasmodium falciparum* was also defective in microneme secretion. However, the major effect was on invasion, suggesting microneme secretion is dispensable for *Plasmodium* egress.

The lytic replication cycle is central to the pathology of apicomplexan diseases such as malaria caused by *Plasmodium* spp. and toxoplasmosis caused by *Toxoplasma gondii*. Motility of parasites between host cells, within which replication occurs, is powered by actinomyosin motors connecting with extracellular substrate through transmembrane adhesion proteins secreted through organelles known as micronemes (1). A pivotal event in triggering motility is the release of Ca²⁺ from compartments within the parasite, which activates myosin and triggers microneme secretion (2). Recently, calcium-dependent protein kinases required for egress were identified in *Plasmodium falciparum*, PfCDPK5, and *Toxoplasma*, TgCDPK1 (3, 4).

To investigate this critical process we used temperature sensitive mutants in the lytic cycle of *Toxoplasma* by chemical mutagenesis (5). Upon phenotype induction, mutant F-P2 displayed a reduced invasion competency (Fig. 1A) and complete inability to egress (6), but

[†]To whom correspondence should be addressed. mduraisi@hsph.harvard.edu, marth@bc.edu, and gubbelsj@bc.edu.

*These authors contributed equally

Supporting Online Material

Materials and Methods

Figs. S1 to S12

Tables S1 to S2

Movies S1 to S4

References (29–55)

intracellular growth progresses normally (6). Three distinct Ca^{2+} -dependent events are required for egress and invasion: extrusion of the apical conoid, motility, and microneme secretion. Conoid extrusion in F-P2 was indistinguishable from wild-type parasites (Fig. 1B, S1, and S5C) (7–9). Motility was assessed by video microscopy and the incidence of the three motility modes of *Toxoplasma* tachyzoites (circular and helical gliding, and twirling; movies S1–3) were scored (9, 10). Under restrictive conditions no circular or helical gliding was observed for F-P2 (Fig. 1C). Rather, induced F-P2 displayed twirling and shuffling, a distinct motility mode wherein parasites abruptly move back and forward (Fig. 1C, movie S4), previously observed with inhibitors of *Toxoplasma* invasion (11), and in certain *T. gondii* (12) and *P. falciparum* sporozoite (13, 14) microneme protein knock-outs. With no net change in parasite position, neither twirling nor shuffling is thought to be effective in invasion or egress. We also assessed microneme release upon increasing the intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) (8, 9, 15). No Mic2 microneme protein release was detected under restrictive conditions for F-P2, but we readily detected dense granule protein release (Gra1) and Mic2 release in the controls (Fig. 1D, S2). Constitutive microneme secretion was also not detected (16) (Fig. 1D). Impaired microneme secretion did not appear to be caused by a defect in microneme formation, morphology, or organelle number because these features were normal (Fig. 1E and S3). Thus, impaired microneme secretion in F-P2 apparently results in parasites unable to provide the traction required for productive motility.

Although other *Toxoplasma* genotypes in chemical mutants have been successfully mapped by genetic complementation (5), we were unable to complement F-P2. We set out to identify the etiological mutation with whole genome mutational profiling using high-throughput sequencing (Fig. 2, Table S1) (17). We identified 31 validated single nucleotide polymorphisms (SNPs) between parent and mutant lines: 8 are in coding regions, 6 intronic and 19 intergenic (table S1). To identify the sole gene responsible for the microneme secretion defect we focused on gene TGGT1_049850, which contains Ca^{2+} -dependent membrane binding C2 domains (18, 19) and harbors a mis-sense mutation (T to C, encoding Phe to Ser) at position 124 (fig. S4). Complementation with cosmid PSBMG64 (9), which spans the entire wild-type locus, resulted in completely restored F-P2 growth (Fig. 3A, S5A), secretion of micronemes (Fig. 3B and S5C), egress (fig. S5B), and largely restored the motility defect (Fig. 3C). To confirm that the point mutation in TGGT1_049850 was solely responsible for the phenotype we complemented with both wild-type and F-P2 mutant cDNA cloned into *T. gondii* expression plasmids. As anticipated, the wild-type allele restores F-P2 growth at 40°C whereas the mutant allele does not (Fig. 3D).

Gene TGGT1_049850 encodes a predicted protein of 1990 amino acids (fig. S4). Orthologs are strongly conserved across the Apicomplexa and some ciliates (e.g. *Paramecium tetraurelia*; Fig. 4A). Motif searches and sequence alignments (9) identified 4 conserved sequence blocks, with block 2 containing a tandem C2 domain, as well as extensive coiled-coil domains in the C-terminus of all orthologs (Fig. 4A). The mutated Phe124 lies in block 1 and is conserved across all orthologs suggesting this residue is critical for function (Fig. 4A, S4). Block 1 appears to be a degenerate C2 domain since a C2 domain was identified just above the consensus cut-off in the *Eimeria tenella* and *Paramecium tetraurelia* orthologs.

Tandem C2 domain proteins function in Ca^{2+} -mediated exocytosis [e.g. neurotransmitter release (20)] and encompass proteins with a transmembrane domain, e.g. synaptotagmin, whereas others lack such a domain, e.g. the double C2 (DOC2) proteins. The C2 domains in cytosolic DOC2A and DOC2B proteins bind Ca^{2+} , facilitating SNARE protein-dependent membrane fusion of secretory vesicles with the plasma membrane (20, 21). We named our protein TgDOC2.1 by taking into account the primary structure and the orthologous function of TGGT1_049850 in microneme secretion. Structural prediction suggests that TgDOC2.1 can bind Ca^{2+} ions through conserved Asp residues in the C2 domains (fig. S6) (22).

TgDOC2.1 does not contain additional recognizable domains to provide further mechanistic insights such as interactions with Munc18, Munc13 or SNARE proteins, though C2 domains could directly fulfill such role.

To determine if the mechanism in *Toxoplasma* is conserved, we generated a *P. falciparum* line allowing inducible regulation of the orthologous DOC2.1 gene, via genetic fusion of a destabilizing domain (DD) to the PfDOC2.1 C-terminus (PFL2110c) (fig. S7). The DD fusion protein is stabilized by the synthetic ligand Shld1 and targeted for degradation in absence of Shld1 (3, 23). PfDOC2.1 levels were reduced by 57+/-13% in the absence of Shld1 (Fig. 4B, S8). To test the effect of PfDOC2.1 on growth, we optimized conditions for measuring parasite replication over multiple cycles by varying initial parasitemia and hematocrit (fig. S9). PfDOC2.1-deficient parasites exhibited an 87% decrease in parasitemia over three cycles (Fig. 4C). D10-PfCDPK4-DD parasites, with an inducible knockdown in PfCDPK4 replicating equally in the presence or absence of Shld1 (3), were used as a control. At this level of PfDOC2.1 knockdown, parasite development within a single asexual cycle through the schizont stage and the number of merozoite nuclei per segmented schizont was unchanged and accumulation of unruptured schizonts was not observed (fig. S10). To evaluate the contribution of PfDOC2.1 to parasite invasion, parasites were cultured in the absence of Shld1 from the ring stage to the schizont stage, and divided into two populations to complete invasion with or without Shld1 present (Fig. S11). Invasion was decreased 34+/-9% compared to parasites rescued by the addition of Shld1 and restoration of PfDOC2.1 levels (Fig. 4D). This decreased invasion efficiency was sufficient to explain the decreased replication rate. To directly identify a defect in microneme secretion, we examined the release of parasite proteins into the supernatants following re-invasion. In the absence of Shld1, release of PfEBA-175, a microneme protein, into the supernatant was decreased by 44+/-14% (Fig. 4E,F and S12A) when normalized for the amount of PfSERA5, a parasitophorous vacuole protein, but the relative use of alternative invasion pathways was unaffected by PfDOC2.1 levels (fig. S12B), suggesting a global defect in invasion secondary to incomplete microneme discharge.

Thus TgDOC2.1 constitutes a second level of Ca²⁺-dependent control of *Toxoplasma* microneme secretion, in addition to the recently identified calcium dependent protein kinase, TgCDPK1 (4). Because DOC2.1 likely facilitates membrane fusion, it probably acts downstream of the CDPKs. Conservation of DOC2.1 and Ca²⁺-dependent secretory organelles in ciliates indicates the shared ancestry of micronemes (24, 25). Although PfDOC2.1 knock-down in *P. falciparum* has a clear effect on invasion at this level of knock-down it did not result in an egress defect, suggesting that *Plasmodium* merozoites may rely on microneme secretion for invasion, but on qualitatively different exome secretion for egress from the erythrocyte (26). However, in *Toxoplasma* mutant F-P2 both invasion and egress were defective, because microneme secretion plays a central role in both these processes (27), highlighting divergent roles for secretory organelles in egress between these organisms. This multi-layered Ca²⁺-mediated control of microneme secretion may underscore tight temporal regulation, and this mechanism appears ancestral to the ciliates and the Apicomplexa.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

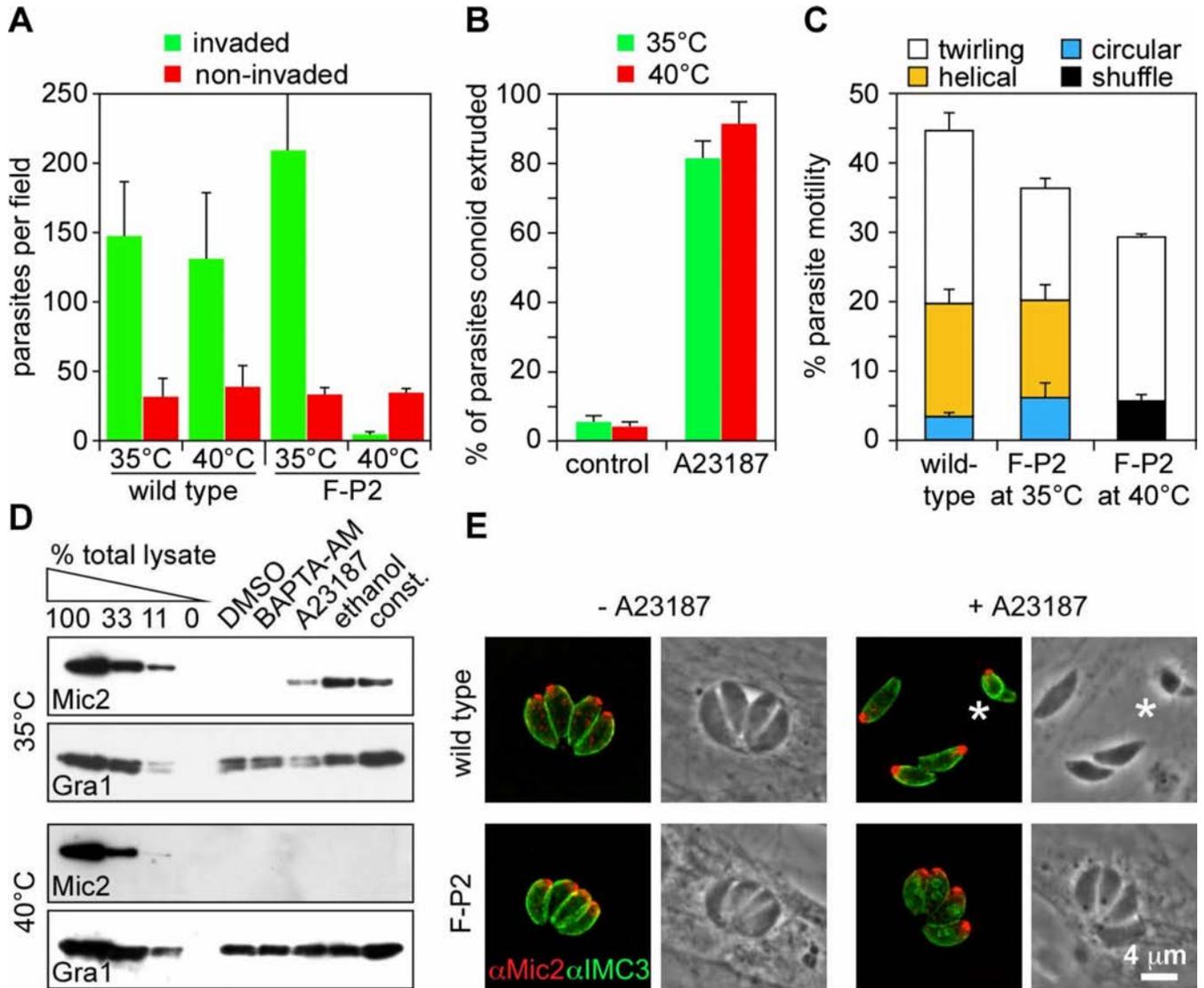
We thank C. Nussbaum and C. Russ of the "Broad Sequencing Platform", D. Sibley, J.-F. Dubremetz, M.-F. Cesbron-Delauw, J.-C. Doury, and M. Makler (Flow Inc.) for kindly sharing reagents. F-P2 was originally generated in the laboratory of B. Striepen. This work was supported by National Institutes of Health grants

AI081220 to MJG and GTM, AI057919 and AI088314 to MTD, HG004719 to GTM, AI087874 to JDD, a Knights Templar Eye Foundation Inc. grant to ST, a Wellcome Trust equipment grant to DJPF, a Burroughs Wellcome Fund New Investigator in the Pathogenesis of Infectious Disease Fellowship to MTD, and an American Heart Association Scientist Development Grant (0635480N) to MJG. *Toxoplasma gondii* GT1 sequence and annotation from L. Caler at the J. Craig Venter Institute. Sequence reads have been deposited at NCBI sequence read archive under accession number SRA046023.

References and Notes

1. Baum J, Gilberger TW, Frischknecht F, Meissner M. Host-cell invasion by malaria parasites: insights from Plasmodium and Toxoplasma. Trends Parasitol. 2008 Dec.24:557. [PubMed: 18835222]
2. Nagamune K, Moreno SN, Chini EN, Sibley LD. Calcium regulation and signaling in apicomplexan parasites. Subcell Biochem. 2008; 47:70. [PubMed: 18512342]
3. Dvorin JD, et al. A plant-like kinase in Plasmodium falciparum regulates parasite egress from erythrocytes. Science. 2010 May 14.328:910. [PubMed: 20466936]
4. Lourido S, et al. Calcium-dependent protein kinase 1 is an essential regulator of exocytosis in Toxoplasma. Nature. 2010 May 20.465:359. [PubMed: 20485436]
5. Gubbels MJ, et al. Forward Genetic Analysis of the Apicomplexan Cell Division Cycle in Toxoplasma gondii. PLoS Pathog. 2008 Feb 15.4:e36. [PubMed: 18282098]
6. Eidell KP, Burke T, Gubbels MJ. Development of a screen to dissect Toxoplasma gondii egress. Mol Biochem Parasitol. 2010 Jun.171:97. [PubMed: 20227445]
7. Gonzalez Del Carmen M, Mondragon M, Gonzalez S, Mondragon R. Induction and regulation of conoid extrusion in Toxoplasma gondii. Cell Microbiol. 2009 Jun.11:967. [PubMed: 19416276]
8. Carruthers VB, Moreno SN, Sibley LD. Ethanol and acetaldehyde elevate intracellular [Ca²⁺] and stimulate microneme discharge in Toxoplasma gondii. Biochem J. 1999 Sep 1.342(Pt 2):379. [PubMed: 10455025]
9. (Materials and methods are available as supporting material on Science Online.) *Materials and methods are available as supporting material on Science Online.*
10. Hakansson S, Morisaki H, Heuser J, Sibley LD. Time-lapse video microscopy of gliding motility in Toxoplasma gondii reveals a novel, biphasic mechanism of cell locomotion. Mol Biol Cell. 1999 Nov.10:3539. [PubMed: 10564254]
11. Carey KL, Westwood NJ, Mitchison TJ, Ward GE. A small-molecule approach to studying invasive mechanisms of Toxoplasma gondii. Proc Natl Acad Sci U S A. 2004 May 11.101:7433. [PubMed: 15123807]
12. Huynh MH, Carruthers VB. Toxoplasma MIC2 is a major determinant of invasion and virulence. PLoS Pathog. 2006 Aug.2:e84. [PubMed: 16933991]
13. Hegge S, et al. Multistep adhesion of Plasmodium sporozoites. FASEB J. 2010 Jul.24:2222. [PubMed: 20159960]
14. Munter S, et al. Plasmodium sporozoite motility is modulated by the turnover of discrete adhesion sites. Cell Host Microbe. 2009 Dec 17.6:551. [PubMed: 20006843]
15. Lovett JL, Marchesini N, Moreno SN, Sibley LD. Toxoplasma gondii microneme secretion involves intracellular Ca(2+) release from inositol 1,4,5-triphosphate (IP(3))/ryanodine-sensitive stores. J Biol Chem. 2002 Jul 19.277:25870. [PubMed: 12011085]
16. Wetzel DM, Chen LA, Ruiz FA, Moreno SN, Sibley LD. Calcium-mediated protein secretion potentiates motility in Toxoplasma gondii. J Cell Sci. 2004 Nov 15.117:5739. [PubMed: 15507483]
17. Smith DR, et al. Rapid whole-genome mutational profiling using next-generation sequencing technologies. Genome Res. 2008 Sep 4.
18. Martens S. Role of C2 domain proteins during synaptic vesicle exocytosis. Biochem Soc Trans. 2010 Feb.38:213. [PubMed: 20074062]
19. Cho W, Stahelin RV. Membrane binding and subcellular targeting of C2 domains. Biochim Biophys Acta. 2006 Aug.1761:838. [PubMed: 16945584]
20. Friedrich R, Yeheskel A, Ashery U. DOC2B, C2 domains, and calcium: A tale of intricate interactions. Mol Neurobiol. 2010 Feb.41:42. [PubMed: 20052564]

21. Groffen AJ, et al. Doc2b is a high-affinity Ca²⁺ sensor for spontaneous neurotransmitter release. *Science*. 2010 Mar 26.327:1614. [PubMed: 20150444]
22. Martens S, McMahon HT. Mechanisms of membrane fusion: disparate players and common principles. *Nature reviews*. 2008 Jul.9:543.
23. Banaszynski LA, Chen LC, Maynard-Smith LA, Ooi AG, Wandless TJ. A rapid, reversible, and tunable method to regulate protein function in living cells using synthetic small molecules. *Cell*. 2006 Sep 8.126:995. [PubMed: 16959577]
24. Erxleben C, Plattner H. Ca²⁺ release from subplasmalemmal stores as a primary event during exocytosis in Paramecium cells. *J Cell Biol*. 1994 Nov.127:935. [PubMed: 7525605]
25. Leander BS, Keeling PJ. Morphostasis in alveolate evolution. *trends in Ecology and Evolution*. 2003; 18:395.
26. Koussis K, et al. A multifunctional serine protease primes the malaria parasite for red blood cell invasion. *Embo J*. 2009 Mar 18.28:725. [PubMed: 19214190]
27. Kafsack BF, et al. Rapid membrane disruption by a perforin-like protein facilitates parasite exit from host cells. *Science*. 2009 Jan 23.323:530. [PubMed: 19095897]
28. Marth GT, et al. A general approach to single-nucleotide polymorphism discovery. *Nat Genet*. 1999 Dec.23:452. [PubMed: 10581034]

**Fig. 1.**

Mutant F-P2 has a microneme secretion defect. (A) Red-green invasion assays were performed on the 2F-1-YFP2 wild-type and F-P2 mutant parasite lines. Parasites were phenotypically induced for 24 hrs at the restrictive temperature (40°C). Averages of four independent experiments + SD are shown. (B) Conoid extrusion of Ca²⁺-ionophore (A23187) induced or vehicle control treated parasites was determined for parasites grown at 35°C or 40°C. Averages of three independent experiments + SD are shown. (C) Incidence of various motility modes determined by video microscopy over 1 min for wild-type (parent 2F-1-YFP2) and F-P2 parasites at conditions as indicated. Averages of four independent experiments + SEM are shown. (D) Microneme secretion of F-P2 parasites measured by western blot detection of Mic2 protein released in the supernatant upon various stimuli and vehicle control (DMSO). “const.” represents uninduced, constitutive secretion over a 60 min period. Gra1 serves as loading control. (E) IFA of Mic2 and IMC3 (marking the peripheral cytoskeleton) of wild-type and F-P2 at 40°C with or without ionophore stimulation shows micronemes are intact in F-P2. Phase images show vacuolar membrane is intact in F-P2 at 40°C. Asterisk marks egressing parasite.

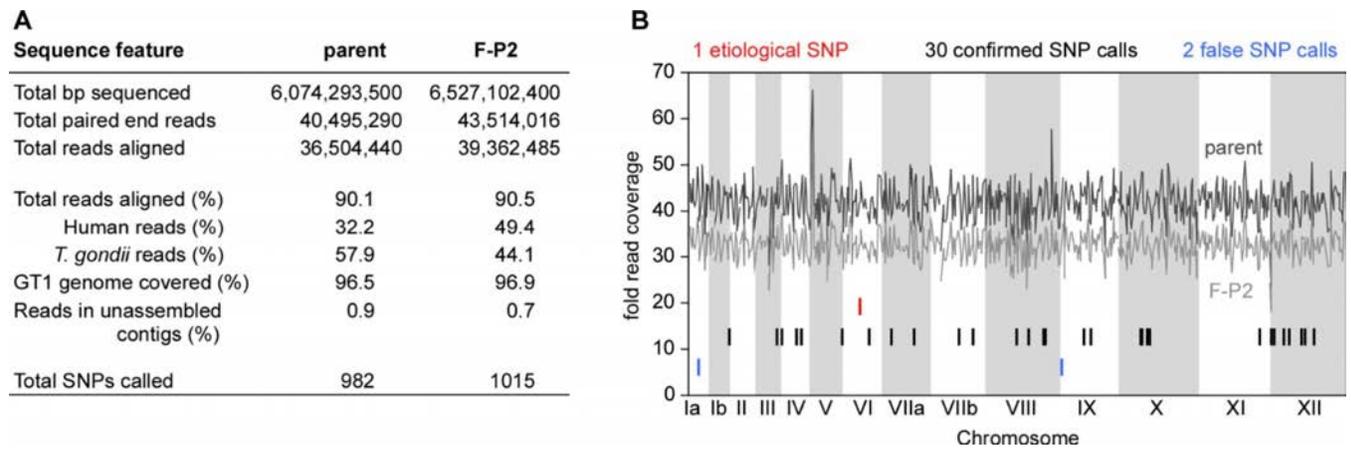


Fig. 2.

Results of paired-end Illumina re-sequencing of parent and F-P2 genomes. **(A)** Sequencing and alignment statistics. Genomic DNA of F-P2 and its parent were sequenced to >30-fold coverage on an Illumina GA2 instrument. Sequence reads were aligned to the closely related GT1 strain reference genome using the MOSAIK read mapper (9). Coverage was sufficient to call sequence differences between F-P2 and RH in >95% of the GT1 reference. Using the FreeBayes variation calling program (28) and appropriate filtering, 982 single nucleotide polymorphisms (SNPs) are shared between the two samples and 33 are unique to F-P2. **(B)** Read coverage across the chromosomes of parent and F-P2. Fold coverage is averaged over 100 kb windows. The chromosomal localization of the 33 called SNPs between parent and F-P2 are shown, differentiated by confirmed and false SNP calls. 31 of the latter were positively confirmed by Sanger sequencing. 77% were mutation of an A/T base, corroborating the proclivity of ENU toward AT:GC and AT:TA substitutions when compared to the rate of 45% of the SNPs shared between the RH and GT1 strains.

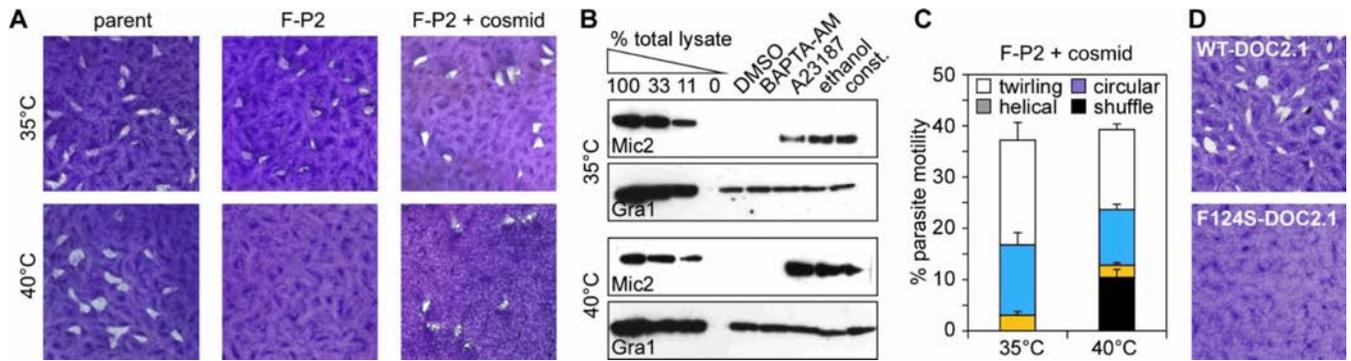
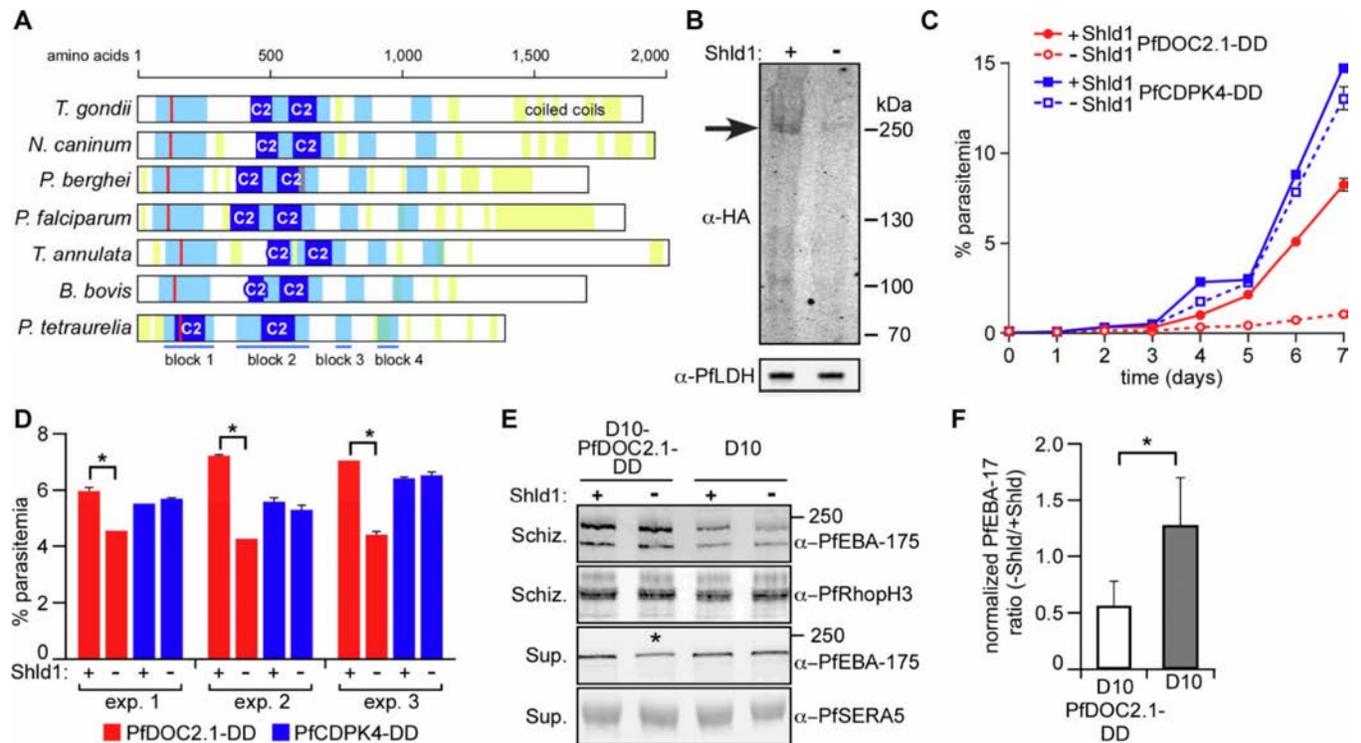


Fig. 3. Genetic complementation of F-P2 with cosmid PSBMG64 restores the wild-type phenotype. **(A)** Plaque assays of parent line (2F-1-YFP2), F-P2 and complemented mutant as indicated. **(B)** Microneme secretion of complemented F-P2 parasites; see legend Fig. 1D. **(C)** Incidence of various motility modes determined by video microscopy over 1 min at conditions as indicated. Averages of four independent experiments shown + SEM. **(D)** Plaque assays representing F-P2 complementation with plasmids expressing cDNA encoding the wild-type TgDOC2.1 allele (top) or the F-P2 mutant allele (bottom).

**Fig. 4.**

DOC2.1 has a conserved role in microneme secretion. **(A)** Schematic of DOC2.1 orthologs in Apicomplexa and ciliate *Paramecium tetraurelia*. Four conserved blocks are color coded in light blue; C2 domains in dark blue; coiled-coil regions in yellow. The red line marks the conserved phenylalanine mutated to serine in F-P2. **(B)** Lysates from D10-PfDOC2.1-DD parasites cultured +/- Shld1 probed with antibody to hemagglutinin (HA) (PfDOC2.1; just over 250 kDa, arrow), or antibody to PfLDH (loading control). Normalized PfDOC2.1 ratio (-/+Shld1): 0.43 +/- 0.13. **(C)** Replication curves of D10-PfDOC2.1-DD parasites maintained +/- Shld1 (mean ± range, N=2). **(D)** D10-PfDOC2.1-DD parasites were maintained without Shld1 from early ring stage to segmented schizont stage then incubated an additional 8–12 hours +/- Shld1, resulting newly invaded rings were counted by FACS (mean ± SD, N=3 [for D10-CDPK4-DD in experiment 3, N=1], three separate experiments, * p<0.001, t-test). **(E)** Immunoblots evaluating the release of indicated parasite invasion ligands. Synchronized ring-stage cells were incubated +/- Shld1. Supernatants of ruptured schizonts lysates (saponin) were collected. The asterisk marks 44±14% decrease in PfEBA-175 release from D10-PfDOC2.1-DD parasites. **(F)** Mean ratio of PfEBA-175 released into supernatant (N=4 biological replicates; +95% CI, *p < 0.01, t-test).