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

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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 Ca^{2+}-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 Ca^{2+} 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

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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 ($[Ca^{2+}]_i$) (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 tetrauarelia*; 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 Ca^{2+}-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 Ca^{2+}-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 exoneme 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 Ca^{2+}-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.

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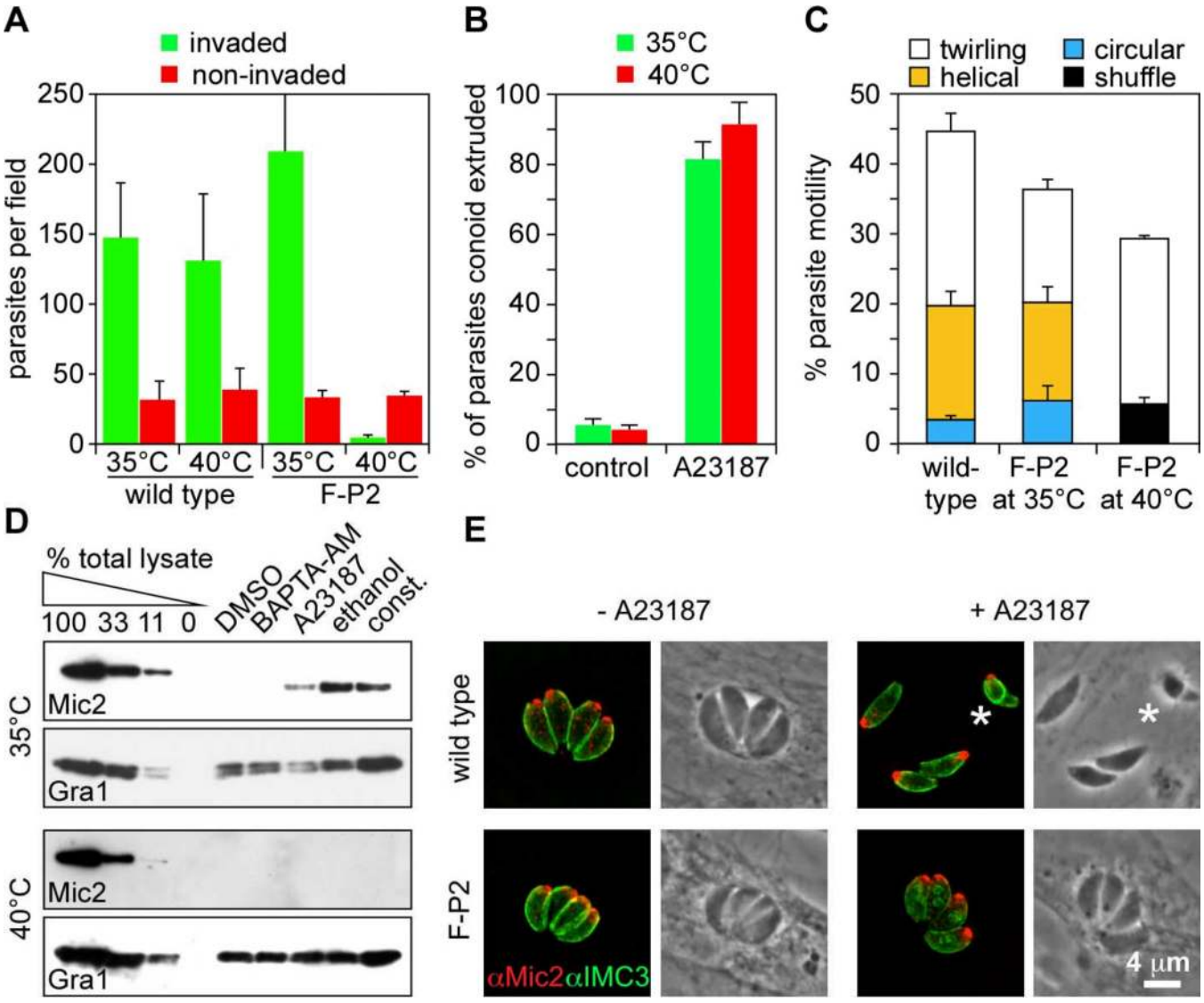

Fig. 1.

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 Ca^{2+}-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.

A

Sequence feature	parent	F-P2
Total bp sequenced	6,074,293,500	6,527,102,400
Total paired end reads	40,495,290	43,514,016
Total reads aligned	36,504,440	39,362,485
Total reads aligned (%)	90.1	90.5
Human reads (%)	32.2	49.4
T. gondii reads (%)	57.9	44.1
GT1 genome covered (%)	96.5	96.9
Reads in unassembled contigs (%)	0.9	0.7
Total SNPs called	982	1015

B

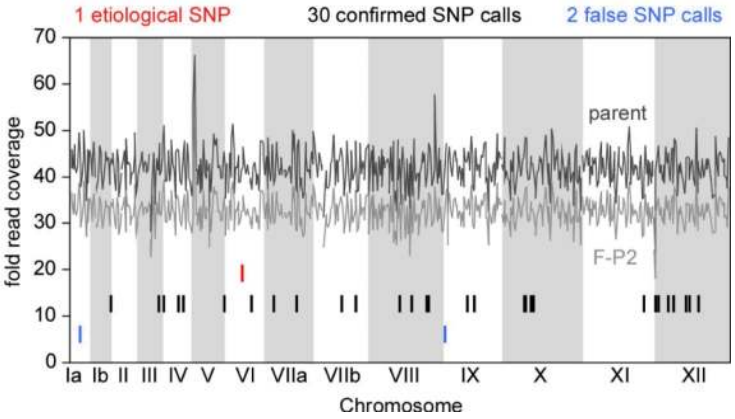

Fig. 2.

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.

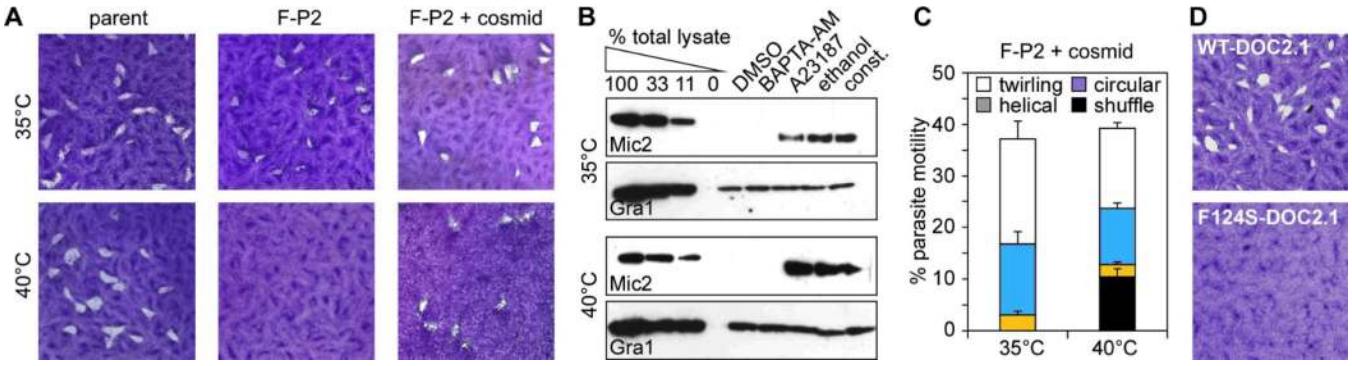

Fig. 3.

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).

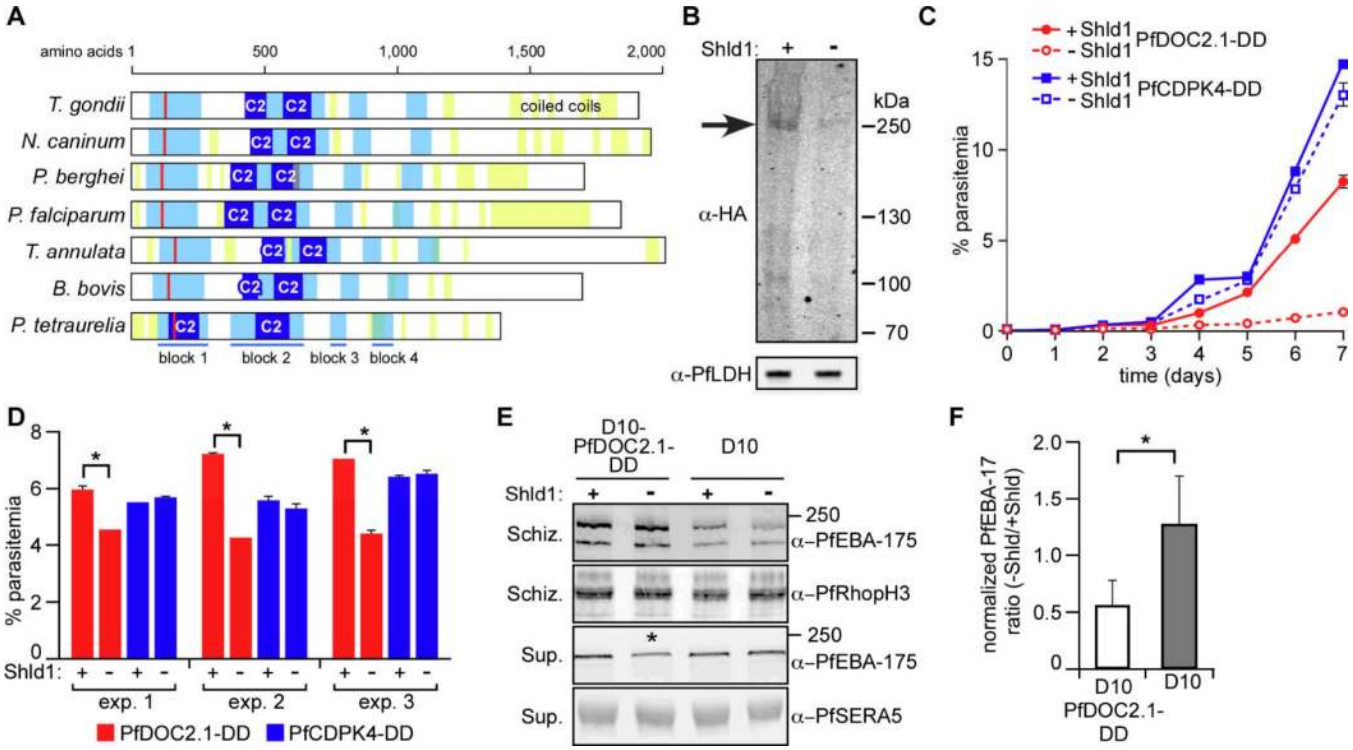

Fig. 4.

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).