Intramembrane cleavage of AMA1 triggers Toxoplasma to switch from an invasive to a replicative mode

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Abstract

Apicomplexan parasites invade host cells and immediately initiate cell division. The extracellular parasite discharges transmembrane proteins onto its surface to mediate motility and invasion. These are shed by intramembrane cleavage, a process associated with invasion but otherwise poorly understood. Functional analysis of Toxoplasma rhomboid 4, a surface intramembrane protease, by conditional overexpression of a catalytically inactive form produced a profound block in replication. This was completely rescued by expression of the cleaved cytoplasmic tail of Toxoplasma or Plasmodium apical membrane antigen 1 (AMA1). These results reveal an unexpected function for AMA1 in parasite replication and suggest that invasion proteins help to promote parasite switch from an invasive to a replicative mode.

Reference


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Intramembrane Cleavage of AMA1 Triggers *Toxoplasma* to Switch from an Invasive to a Replicative Mode

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MATERIAL AND METHODS

Reagents and parasite culture
Restriction enzymes were purchased from New England Biolabs and secondary antibodies for Western blots and IFA from Molecular Probes. T. gondii tachyzoites (RHΔHX) were grown in human foreskin fibroblasts (HFF) in Dulbecco’s Modified Eagle’s Medium (DMEM, GIBCO) supplemented with 5% fetal calf serum (FCS), 2mM glutamine and 258 µg/ml gentamicin.

Antibodies
The α-Ty tag BB2, α-myc tag monoclonal antibody 9E10, α-PRF, α-GAP45, α-SAG1 and α-GRA3 antibodies were described previously (S1). The polyclonal antibodies α-ROM4Nt and α-MIC2 and monoclonal antibodies 1B10 α-ROP7, 11G8 α-Atrx1 were previously used in (S2-5), respectively. Monoclonal antibodies 5F4 (α-F1 ATPase beta subunit) and 7E8 (anti-ISPI) (S6) and 1B10 and 11G8 were kindly provided by P. Bradley. The affinity purified antibody α-MORN1 was previously used in (S7) and was kindly provided by M. Gubbels. Secondary α-rabbit (Molecular Probes G21234) and α-mouse (Molecular Probes G21040) antibodies coupled to HRP were used to detect proteins on Western blots. Appropriate secondary antibodies from Molecular Probes (Alexa Fluor) were used for IFA.

Cloning of DNA constructs
For expression of ROM1 and ROM4 in T. gondii as N-terminal ddFKBPmyc-epitope tagged fusions, mycROM1 and mycROM4 from pT8mycROM1 and pT8mycROM4 plasmids (S8) were digested with NsiI and PacI and cloned into the pT8ddmycGFP vector, which drives expression of N-terminal ddFKBP-myc tagged fusions under control of tubulin promoter, producing pT8ddmycROM1 and pT8ddmycROM4, respectively. The pT8ddmycGFP plasmid was obtained by digesting the plasmid pT8ddYFP (S9) (kindly provided by M. Meissner) with EcoRI and NsiI and cloning the ddFKBP sequence into the same sites in the pT8mycGFPty vector (S10). The primers 1703 and 1704 and 1516 and 1517 (Table S1) were used in a site-directed mutagenesis reaction using the commercial QuikChange II Site-Directed Mutagenesis Kit (Stratagen) according to the manufacturer’s instructions, to mutate the catalytic
Ser residues to Ala in the pT8ddmycROM1 and pT8ddmycROM4 plasmids, producing pT8ddmycROM1S-A and pT8ddmycROM4S-A, respectively. Digestion of pT8ddmycROM4 and pT8ddmycROM4S-A with NaeI and XbaI and cloning into the same sites in the p30/11 plasmid (S11) produced plasmids pT8ddmycROM4(DHFR) and pT8ddmycROM4S-A(DHFR), respectively. The plasmids pT8ddmycHisROM4 and pT8ddmycHisROM4(DHFR) were obtained by amplifying from pT8ddmycHis with primers 2576 and 2577 and cloning into the same sites in pT8ddmycROM4 or pT8ddmycROM4(DHFR), respectively.

To express Ty-epitope tagged ROM4, ROM4 and ROM4S-A were amplified from pT8ddmycROM4 or pT8ddmycROM4S-A, respectively, by PCR using the primers 1483 and 1484, and cloned between EcoRI and NsiI in the pT8mycGFPty vector. To generate ddFKBP fusions, ROM4Ty and ROM4S-A Ty were cloned between MfeI and PacI in the pT8ddmycGFP vector, generating pT8ddmycROM4Ty and pT8ddmycROM4S-A Ty, respectively.

To express the AMA1 and MIC2 C-terminus as N-terminal myc-His tagged ddFKBP fusions, AMA1 and MIC2 were amplified from tachyzoite cDNA by PCR with primers 1080 and 2614 and 2945 and 2946, respectively, and cloned between NsiI and PacI in pT8ddmycHisROM4 or pT8ddmycHisROM4(DHFR), producing pT8ddmycHisAMA1 and pT8ddmycHisAMA1(DHFR) or pT8ddmycHisMIC2 and pT8ddmycHisMIC2(DHFR), respectively. Primers 1830 and 1831 were used in a site-directed mutagenesis reaction to mutate the FW motif into AA in pT8ddmycHisAMA1 and pT8ddmycHisAMA1(DHFR), generating pT8ddmycHisAMA1FW-AA and pT8ddmycHisAMA1FW-AA (DHFR). AMA1535-570 and AMA1504-549 were amplified with primers 1080 and 3129 or 2614 and 3131, respectively, and cloned between NsiI and PacI in pT8ddmycHisROM4 or pT8ddmycHisROM4(DHFR), producing pT8ddmycHis AMA1535-570 and pT8ddmycHis AMA1504-549 (DHFR) or pT8ddmycHisAMA1504-549 (DHFR), respectively. The PfAMA1 tail was expressed as a synthetic gene (CGGATGCATAAGCGAAGGGCAACCGGAGAAGTACGAC AAGATGGACGACGCAGAGACTACGGCAAGTCGAATCCTCGCAGCAACGACG AGATGCTCGACCCGGAGGCTGTCGTTCTGGGCGAGGAAAGCGCGGCCT GCACAGACCCGGTGTCCTACGGTACCTACTGATTAATAGGC) and cloned between NsiI and PacI in pT8ddmycHisROM4 or pT8ddmycHisROM4(DHFR), producing pT8ddmycHisPfAMA1 or
pT8ddmycHisPfAMA1(DHFR). Primers 3140 and 3141 were used in a site-directed mutagenesis reaction to mutate the Ser610 residue to Ala, generating pT8ddmycHisPfAMA1_S-A and pT8ddmycHisPfAMA1_S-A(DHFR).

Parasites transfection and selection of clonal stable lines
Parasite transfection was performed by electroporation as previously described (SI2). The HXGPRT gene was used as a positive selectable marker in the presence of mycophenolic acid and xanthine, as described before (SI3). Briefly, 5×10^7 freshly released RHΔHX parasites were resuspended in cytomix buffer in the presence of 80 µg of linearized plasmid carrying the selectable marker gene and the expression cassette containing the DNA sequences. Parasites were electroporated at 2 kV, 25 mF, 48 V using a BTX electroporator (Harvard biosciences, Holliston, MA, USA) before being added to a monolayer of HFF cells in the presence of mycophenolic acid (25 mg/mL) and xanthine (50 mg/mL) and were then cloned by limiting dilution in 96-well microtiter plates following one growth cycle. The same procedure was followed to transfect the ΔAMA1/AMA1-myc, the ddROM4 or the ddROM4_S-A strains, but the DHFR gene was used as a positive selectable marker. Selection with 1 µM pyrimethamine was initiated 24 h after transfection and continued for 7-10 days, after which resistant clones were isolated by limiting dilution.

Transmission electron microscopy
ddROM4_S-A parasites, which had undergone or not a 12 h pre-treatment with 0.5 µM Shld-1, were used to infect a host cell layer ± 0.5 µM Shld-1. Samples were collected at 18, 24 and 36 h post-infection and processed for electron microscopy using routine techniques. Briefly, parasite pellets were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer, post-fixed in osmium tetroxide, dehydrated in ethanol and treated with propylene oxide prior to embedding in Spurr’s epoxy resin. Thin sections were stained with uranyl acetate and lead citrate prior to examining in a Jeol 1200EX electron microscope.

Uracil incorporation assay
3×10^6 freshly lysed parasites were seeded into 24-wells and incubated ±0.5 µM Shld-1. The extracellular parasites were removed by wash 3 h later and the medium was
supplemented with 3 μCi ±0.5 μM Shld-1 per well. After a further 24 h incubation, parasite nucleic acid was collected on a filter membrane by 1% SDS-0.3 N TCA precipitation on ice. The filters were washed twice with 0.3 N TCA and once with 95% ethanol and the number of counts per minute (CPM) was determined in a plate reader. Each condition was performed in triplicate and the assay was repeated two times.

**Western blotting**

2x10⁷ freshly lysed parasites ±0.5 μM Shld-1 treated for 24 h were harvested after complete lysis of the host cells. Protein extracts were prepared in 1xPBS by five consecutive freeze/thaw cycles with intermediate homogenization. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using standard methods: the suspension was mixed with SDS–PAGE-loading buffer and proteins were separated by electrophoresis on a 10-12% polyacrylamide gel. Separated proteins were transferred to a nitrocellulose membrane using a semidry electroblotter. Western blots were performed using anti-Myc mAb 9E10, anti-Ty mAb, anti-ROM4_{NI} polyclonal antibodies (S2) or anti-Profilin polyclonal antibodies (S1) in 5% non-fat milk powder in 1X PBS-0.05% Tween. As secondary antibody, a peroxidase-conjugated goat anti-mouse or anti-rabbit antibody was used (Molecular Probes, Paisley, UK). Bound antibodies were visualized using the ECL system (Amersham Corp).

**IFA and confocal microscopy**

Intracellular parasites grown in HFF were fixed with 4% paraformaldehyde (PAF) or 4% PAF-0.005% glutaraldehyde (PAF/GA) in PBS depending on the antigen to be labeled and processed as previously described (S1). The percentage of parasites undergoing endodyogeny in cultures deficient in micronemes, nuclear, rhoptries or mitochondrion staining was determined on *ddROM4_{S,A}* parasites treated ± 0.5 μM Shld-1 for 24 h. For each condition, at least 100 vacuoles were examined.

**Plaque assays**

Monolayers of HFF grown in 6-well plates were infected with tachyzoites and
incubated for 6-7 days at 37°C, after which they were fixed and stained with Giemsa stain for 10 minutes and washed with water.

**Intracellular Growth assays**

New host cells seeded on 24-well IFA plates were inoculated with freshly egressed parasites pre-treated ± 0.5 µM Shld-1. Parasites were allowed to grow for 24 h ± 0.5 µM Shld-1 before fixation with 4% paraformaldehyde. Double immunofluorescence assays were performed and the number of parasites in at least 100 vacuoles were counted for each condition. Pulse-chase intracellular growth assays were performed in the same way but parasites were treated ± 0.5 µM Shld-1 for the times indicated. For the assays with the ΔAMA1-AMA1myc and RH-2YFP strains, parasites were treated for 24 h ± 1 µM Atc prior to host cell egress. New host cells seeded on 24-well IFA plates were inoculated with freshly egressed parasites ± 1 µM Atc. The extracellular parasites were removed by wash 3 h after host cell inoculation and the resulting intracellular parasites allowed to grow for 24 h before fixation with 4% PAF. IFA was performed and the number of parasites in at least 100 vacuoles were counted for each condition. Results presented are representative of at least three independent experiments.

**Cell invasion assays**

The assay was done using the RH-2YFP strain as an internal standard, as previously described (SI4). Briefly, a confluent 60 mm-dish of HFF was infected with a mixture of the strain of interest and RH-2YFP parasites. Parasites were either treated ± 0.5 µM Shld-1 for 12 h, when intracellular, or for only 6 h (in the case of ddROM4s,Δ), whilst extracellular. The ratio of non-YFP to YFP parasites was determined and the mix of parasites was inoculated into IFA plates. The plate was incubated for 1 h at 37°C and the cells examined by IFA. The total number of parasites and the ratio of YFP to non-YFP vacuoles were counted in at least 100 vacuoles per slide. Each experiment was repeated at least 3 independent times.

**Induced egress assays**

Parasites were grown for 6 h ± 0.5 µM Shld-1. Freshly egressed tachyzoites were used to inoculate new host cell layers. After 30 h of intracellular growth, the infected host cell layers were incubated for 5 min at 37°C with DMEM containing DMSO or
the calcium ionophore A23187 (from *Streptomyces chartreusensis*, Calbiochem). The host cells were fixed with PAF and IFA with α-GAP45 and α-GRA3 antibodies were performed.

**Induced Gliding assays**
Parasites were grown for 12 h ± 0.5 µM Shld-1 before host cell egress. Freshly egressed tachyzoites were pelleted, and resuspended in a calcium-saline solution containing 1 mM ionomycin. The suspension was deposited on coverslips previously coated with Poly-L-Lysine. Parasites were fixed with PAF/GA and IFA using the α-SAG1 antibody were performed to visualize the trails.

**Statistics**
P values were calculated in Excel using the Student’s t-test assuming equal variance, unpaired samples, and using 2-tailed distribution. Means and standard deviations (SD) were also calculated in Excel.
SUPPLEMENTARY TEXT

Previous studies using the Atc-regulated conditional knockout strategy for the study of AMA1 or ROM4 did not detect a role for ROM4 or AMA1 during replication (S15, 16). In (S16), replication of an Atc-regulated conditional knockout strain of AMA1 (Δama1/AMA1-myc) was examined only 6 h after expression of the inducible copy of AMA1 (AMA1-myc) had become undetectable. Reexamination of the phenotype of the Δama1/AMA1-myc line found that a cell division defect became evident 48 h post-treatment (Fig. S8A), confirming a role for AMA1 in replication. The lack of a more profound defect may be explained by incomplete depletion of AMA1-myc. Expression of ddROM4S,A in these parasites (Figs. S8B-C) led to a defect in parasite division and invasion similar to that seen with wild type parasites (Figs. S8D-F). In (S15), the failure to observe a replication defect may have been the result of an inherent limitation of the system; if the function of ROM4 in parasite division depends on a limited amount of substrate cleavage, even trace expression of ROM4 could be sufficient to sustain replication. In contrast, our dominant negative strategy was designed to efficiently sequester even low levels of substrate.

Results presented in this study support the notion that ROM4-mediated cleavage of AMA1 signals the switch from an invasive to a replicative mode, and, in T. gondii, a new stimulus is needed at each new replication cycle. In contrast, during Plasmodium erythrocytic cycle, in which daughter parasites are generated simultaneously, only one signaling event may be required at the time of invasion. MIC2 plays an essential function in motility and invasion (S17) and ROM4 cleavage is important for both (S15). Expression of ddMIC2 does not revert the effect caused by expression of ddROM4S,A and AMA1 shedding also takes place on gliding, extracellular parasites, suggesting that ROM4-mediated cleavage is necessary but not sufficient for triggering replication. The model implies that AMA1 or alternatively another substrate is constitutively secreted onto the surface of intracellular parasites, at very low levels. This is supported by secretion assays performed with AMA1 and MIC2 (S18) and microscopic evidence that AMA1 is found on invading parasites (S16, 18, 19); although cleavage during transit of ROM4 to the parasite surface cannot be discounted.
SUPPLEMENTARY FIGURES
Figure S1. Stabilization of ddROM4 in intracellular parasites as a function of time

(A) Kinetics of ddROM4 expression as determined by Western blot of parasites treated with Shld-1 from 30 to 720 min, compared with expression of the endogenous protein. Detection was with antibodies specific to the ROM4 N-terminus (ROM4\text{Nt}).

(B) Kinetics of ddROM4 expression determined by IFA of parasites treated with Shld-1 from 5 to 360 min using α-myc (green) and α-GAP45 (red) antibodies.
(C) $[^3]$H-uracil incorporation into TCA precipitable parasite nucleic acids can be used as measure of growth because in contrast to mammalian cells, T. gondii can directly salvage uracil through UPRT. This assay indicates that the ddROM4$_{S.4}$ parasites are affected in replication when grown with Shld-1 and the ddROM4 parasites grow better in the presence of Shld-1. The y-axis indicates the fold difference in $[^3]$H-uracil incorporation of the Shld-1 treated samples versus the non-treated samples. Data are represented as mean ± SD of three independent experiments.
Figure S2. C-terminal epitope tagging of ddROM4S-A abrogates its dominant negative effect

(A) Expression of both ddROM4Ty and ddROM4S-ATy is efficiently stabilized by treatment with Shld-1 for 12 h as determined by indirect immunofluorescence assay.
(IFA) using $\alpha$-Ty antibodies (green). $\alpha$-GAP45 antibodies (red) were used to stain the parasite. Scale bar, 5 $\mu$m.

(B) Western blot showing stable expression of ddROM4Ty and ddROM4$_{S,A}$Ty ($\alpha$-Ty antibody, top panel) as compared to the expression of endogenous ROM4 ($\alpha$-ROM4$_{Nt}$, middle panel) upon treatment with Shld-1 for 12 h. SAG1 was used as a loading control.

(C) Plaque assays of $ddROM4Ty$ and $ddROM4_{S,A}Ty$ parasites grown for 7 days ± Shld-1.

(D) Intracellular replication assays of $ddROM4Ty$ or $ddROM4_{S,A}Ty$ parasites. Parasites were grown 24 h ± Shld-1 prior to fixation. Data are represented as mean ± SD of three independent experiments.
Figure S3. Assessment of the arrest in replication in ddROM4_{S,A} parasites by IFA
Parasites expressing ddROM4_{S,A} have a normally developed apicoplast (α-ATrx1, green) and basal complexes (α-MORN1, red), but the micronemes were less numerous (α-MIC2, green) and the rhoptries appeared elongated and disorganized (α-ROP7, green). Parasites were treated ± Shld-1 for 24 h prior to fixation. Arrowheads indicate concentration of the micronemes at the apical tip of the parasite or elongation of the rhoptries. α-GAP45 or α-myc antibodies were used to stain the parasites. Scale bar, 5 μm.
Figure S4 Shld-1 treatment of ddROM4S.A parasites affects gliding, host cell invasion and egress

(A) Stabilization of ddROM4S.A expression in extracellular parasites treated for 6 h ± Shld-1 modestly affected host cell invasion, whereas expression of ddROM4 or ddROM1S.A had no effect. The y-axis represents the percentage of intracellular parasites as the mean ± SD compared to the RH-2YFP control as determined in four independent experiments. * indicates a statistically significant reduction (p=0.08) as determined by the Student’s t test.
In vitro gliding motility assay based on the detection of trails using α-SAG1 antibodies by IFA. A trail is indicated by the white arrowhead. Intracellular parasites were treated 12 h ± Shld-1 prior to egress and assay. Ca^{2+}-ionophore (A23187)-induced egress of extracellular *ddROM4* (C) or *ddROM4* (D) parasites following 6 h of treatment ± Shld-1. The parasite surface was stained with α-SAG1 antibodies and α-GRA3 antibodies were used as a marker of the parasitophorous vacuole. Scale bar, 5 μm.
Figure S5 Expression of ddAMA1, ddAMA1$_\text{FW-AA}$, ddPfAMA1 and ddMIC2 in $RH\DeltaHX$, ddROM4 or ddROM4$_S$-$A$ parasites is efficiently regulated by Shld-1

(A) Western-blot of $RH\DeltaHX$ (left), ddROM4 (middle) or ddROM4$_S$-$A$ (right) parasites stably expressing ddAMA1 as detected with $\alpha$-myc. SAG1 was used as loading control.
(B) Western-blot of RHΔHX (left), ddROM4 (middle) or ddROM4S-A (right) parasites stably expressing ddAMA1FW-AA as detected with α-myc. SAG1 was used as loading control.

(C) Western-blot of RHΔHX, ddROM4 or ddROM4S-A parasites stably expressing ddMIC2 as detected with α-myc. SAG1 was used as loading control.

(D) Western-blot of RHΔHX, ddROM4 or ddROM4S-A parasites stably expressing ddPfAMA1 as detected with α-myc. SAG1 was used as loading control.

(E) IFA of RHΔHX, ddROM4 or ddROM4S-A parasites expressing ddAMA1, ddAMA1FW-AA or ddMIC2 treated for 12 h ± Shld-1. The parasites were stained with α-GAP45 (red) and α-myc antibodies (green). Scale bar, 5 µm.
Figure S6 Plaque and intracellular growth assays of RH and ddROM4 parasites expressing ddAMA1, ddAMA1FW-AA, ddPfAMA1 or ddMIC2

Plaque assays and intracellular growth assays of RHΔHX parasites expressing ddAMA1 (A), ddAMA1FW-AA (B), ddPfAMA1 (C) or ddMIC2 (G). Plaque assays and intracellular growth assays of ddROM4 parasites expressing ddAMA1 (D), ddAMA1FW-AA (E), ddPfAMA1 (F) or ddMIC2 (H). Parasites were treated for 7 days
or for 24 h ± Shld-1 prior to fixation for the plaque assays and intracellular growth assays, respectively. Data are represented as mean ± SD as determined in three independent experiments.
Figure S7 Plaque and intracellular growth assays of *ddROM4* parasites expressing *ddAMA1* _DY-AA_, *ddAMA1* _535-570_ or *ddPfAMA1* _S-A_.

Plaque and intracellular growth assays of *ddROM4* parasites expressing *ddAMA1* _DY-AA_ (A), *ddAMA1* _535-570_ (B) or *ddPfAMA1* _S-A_ (C). Parasites were treated for 7 days or for 24 h ± Shld-1 prior to fixation for the plaque assays and intracellular growth assays.
growth assays, respectively. Data are represented as mean ± SD as determined in three independent experiments.

(D) IFA of a mixed population of parasites expressing only ddROM4<sub>S-A</sub> (arrowhead) or ddROM4<sub>S-A</sub> and ddAMA1<sub>FW-AA</sub> (star) treated for 24 h with Shld-1 prior to fixation. Parasites were stained with α-GAP45 (red) and α-myc (green) antibodies. The effects of ddAMA1<sub>FW-AA</sub> expression on parasite replication can be clearly observed.
Figure S8 ΔAMA1-AMA1myc parasites are affected in cell division

(A) Intracellular growth assays of ΔAMA1-AMA1myc (left) and RH-2YFP parasites (right) not Atc treated (-Atc), Atc treated for only the time of the assay (+Atc 24 h) or pre-treated for 24 h with Atc prior to egress and for the time of the assay, in a total of 48 h (+Atc 48 h). The number of parasites per vacuole was counted 24 h after removal of the extracellular parasites (which was done 3 h after invasion). Data are
represented as mean ± SD as determined in three independent experiments. * indicates statistically significant results (*: p=0.09, **: p=0.02) as determined by the Student’s t test.

(B) IFA showing stabilization and down-regulation of ddROM4$_{S-A}$ in ΔAMA1-AMA1myc parasites expressing ddROM4$_{S-A}$ after 12 h of treatment with Shld-1. Parasites were stained with α-GAP45 (red) and α-myc (green) antibodies. Scale bar, 5 µm. The same results were obtained for ΔAMA1-AMA1myc parasites expressing ddROM4.

(C) Western blot assays showing stabilization and down-regulation of ddROM4/ddROM4$_{S-A}$ and AMA1myc after 24 h of treatment with Shld-1 or Atc, respectively. The proteins were detected with α-myc antibodies. SAG1 was used as a loading control.

(D) Plaque assays of ΔAMA1-AMA1myc parasites expressing ddROM4 (top) or ddROM4$_{S-A}$ (bottom) treated ± Shld-1 for 7 days.

(E) Invasion assays of RHΔHX (left) or ΔAMA1-AMA1myc parasites (right) expressing ddROM4$_{S-A}$ ± 6 h of treatment with Shld-1, when extracellular, as compared to the RH-2YFP strain. Data are represented as mean ± SD as determined in three independent experiments. * indicates statistically significant results (*: p=0.08) as determined by the Student t test.

(F) Intracellular growth assays of ΔAMA1-AMA1myc parasites expressing ddROM4 (left) or ddROM4$_{S-A}$ (right) ± 24 h treatment with Shld-1. The number of parasites per vacuole was counted 24 h after invasion. Data are represented as mean ± SD as determined in three independent experiments. * indicates statistically significant results (**: p=0.003 and ***:p=0.001).
SUPPLEMENTARY TABLES

Table S1. List of primers used in the study
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Table S2. Number of parasites per vacuole and percentage of \textit{ddROM4}_{S-A} parasites undergoing endodyogeny at different time points as determined by IFA and EM

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<th>Shld-1</th>
<th>% 1+2par/vac</th>
<th>% 4+par/vac</th>
<th>% endodyogeny</th>
</tr>
</thead>
<tbody>
<tr>
<td>18h</td>
<td>-</td>
<td>84</td>
<td>16</td>
<td>12\textsuperscript{a}</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>99</td>
<td>&lt;1</td>
<td>5\textsuperscript{a}</td>
</tr>
<tr>
<td>24h</td>
<td>-</td>
<td>32</td>
<td>68</td>
<td>21\textsuperscript{a} 40\textsuperscript{b}</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>90</td>
<td>10</td>
<td>4\textsuperscript{a} 10\textsuperscript{b}</td>
</tr>
<tr>
<td>36h</td>
<td>-</td>
<td>c</td>
<td>c</td>
<td>c</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>99</td>
<td>&lt;1</td>
<td>5\textsuperscript{a}</td>
</tr>
</tbody>
</table>

\textsuperscript{a} determined by EM
\textsuperscript{b} determined by IFA
\textsuperscript{c} monolayer destroyed (no counts possible)

Table S3. Percentage of vacuoles containing \textit{ddROM4}_{S-A} parasites with deficiencies in the mitochondrion, micronemes, rhoptries or nucleus as determined by IFA

<table>
<thead>
<tr>
<th>Shld-1</th>
<th>% Mitochondrion</th>
<th>% Micronemes</th>
<th>% Rhoptries</th>
<th>% Nucleus</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>&lt;5</td>
<td>&lt;5</td>
<td>&lt;5</td>
<td>&lt;10</td>
</tr>
<tr>
<td>+</td>
<td>46</td>
<td>34</td>
<td>59</td>
<td>88</td>
</tr>
</tbody>
</table>
REFERENCES