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

Santos, Joana M.; Ferguson, David J P.; Blackman, Michael J.; Soldati-Favre, Dominique

Supporting Online Material for

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

Joana M. Santos, David J. P. Ferguson, Michael J. Blackman, Dominique Soldati-Favre*

*To whom correspondence should be addressed. E-mail: Dominique.Soldati-Favre@unige.ch

Published 23 December 2010 on *Science* Express
DOI: 10.1126/science.1199284

This PDF file includes:

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-ISP1) (*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, myc*ROM1* and myc*ROM4* from pT8mycROM1 and pT8mycROM4 plasmids (*S8*) were digested with *Nsi*I and *Pac*I 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 *EcoR*I and *Nsi*I 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 pT8ddmycROM1$_{S\text{-}A}$ and pT8ddmycROM4$_{S\text{-}A}$, respectively. Digestion of pT8ddmycROM4 and pT8ddmycROM4$_{S\text{-}A}$ with *Nae*I and *Xba*I and cloning into the same sites in the p30/11 plasmid (*S11*) produced plasmids pT8ddmycROM4(DHFR) and pT8ddmycROM$_{S\text{-}A}$(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 *ROM4$_{S\text{-}A}$* were amplified from pT8ddmycROM4 or pT8ddmycROM4$_{S\text{-}A}$, respectively, by PCR using the primers 1483 and 1484, and cloned between *Eco*RI and *Nsi*I in the pT8mycGFPty vector. To generate ddFKBP fusions, *ROM4Ty* and *ROM4$_{S\text{-}A}$Ty* were cloned between *Mfe*I and *Pac*I in the pT8ddmycGFP vector, generating pT8ddmycROM4Ty and pT8ddmycROM4$_{S\text{-}A}$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 pT8ddmycHisAMA1$_{FW\text{-}AA}$ and pT8ddmycHisAMA1$_{FW\text{-}AA}$ (DHFR). AMA1$_{535\text{-}570}$ and AMA1$_{504\text{-}549}$ were amplified with primers 1080 and 3129 or 2614 and 3131, respectively, and cloned between *Nsi*I and *Pac*I in pT8ddmycHisROM4 or pT8ddmycHisROM4(DHFR), producing pT8ddmycHis AMA1$_{535\text{-}570}$ and pT8ddmycHis AMA1$_{535\text{-}570}$ (DHFR) or pT8ddmycHis AMA1$_{504\text{-}549}$ and pT8ddmycHis AMA1$_{504\text{-}549}$ (DHFR), respectively. The PfAMA1 tail was expressed as a synthetic gene (CGGATGCATAAGCGCAAGGGCAACGCGGAGAAGTACGAC
AAGATGGACGAGCCGCAGGACTACGGCAAGTCGAACTCGCGCAACGACG
AGATGCTCGACCCGGAGGCGTCGTTCTGGGGCGAGGAGAAGCGCGCGTC
GCACAGACGCCGGTCCTCATGGAGAAGCCGTACTACTGATTAATTAAGGC
) and cloned between *Nsi*I and *Pac*I 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 Ser_{610} residue to Ala, generating pT8ddmycHisPfAMA1$_{S\text{-}A}$ and pT8ddmycHisPfAMA1$_{S\text{-}A}$ (DHFR).

Parasites transfection and selection of clonal stable lines

Parasite transfection was performed by electroporation as previously described (*S12*). The HXGPRT gene was used as a positive selectable marker in the presence of mycophenolic acid and xanthine, as described before (*S13*). 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$_{S\text{-}A}$* 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$_{S\text{-}A}$ 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

$2x10^7$ 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-$ROM4_{Nt}$ 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 $ddROM4_{S\text{-}A}$ 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 37ºC, 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 (*S14*). 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 $ddROM4_{S\text{-}A)}$, 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 37ºC 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 37ºC 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 IFAusing 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 ddROM4$_{S\text{-}A}$ 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 ddROM4$_{S\text{-}A}$ 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

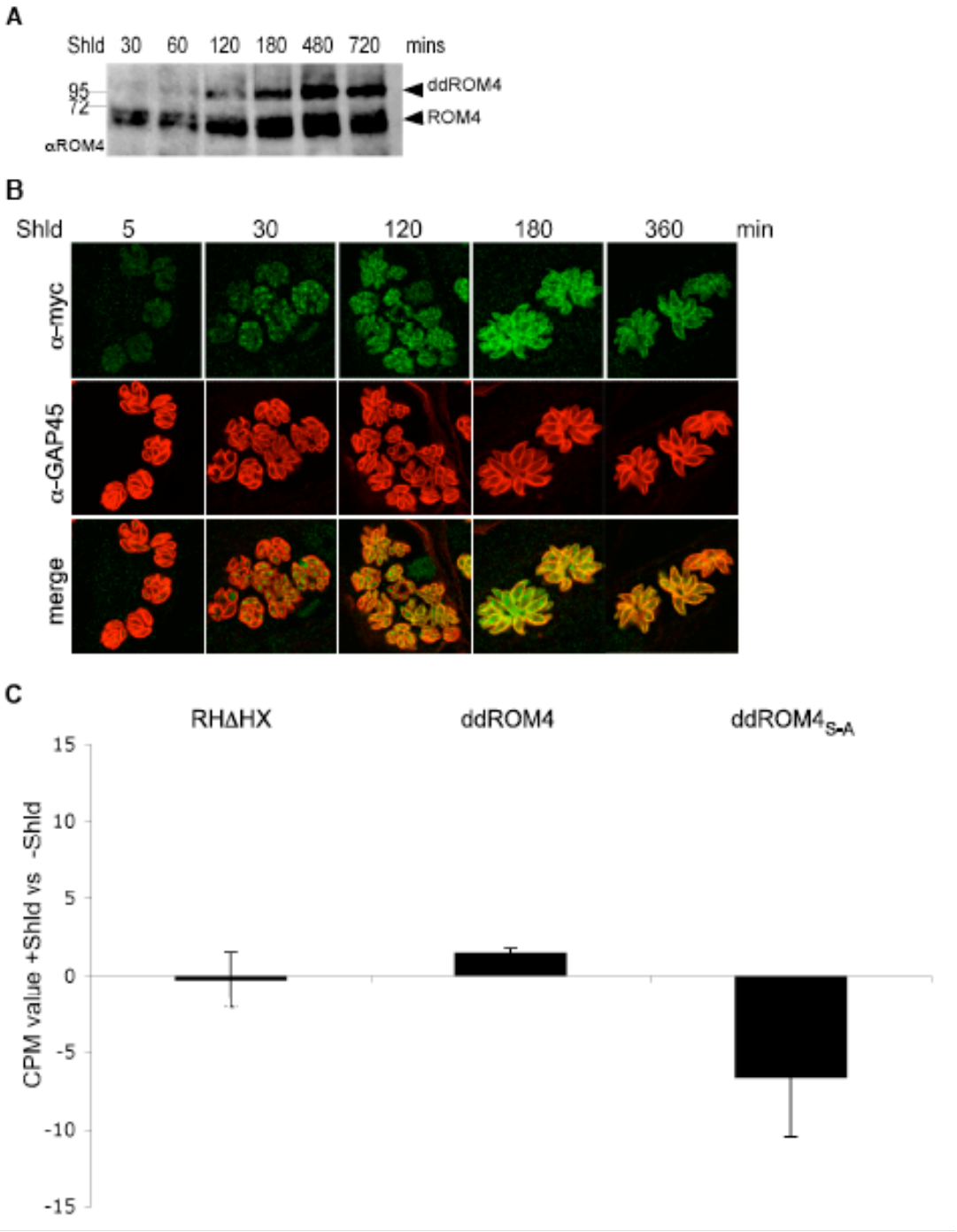

Figure S1. Stabilization of ddROM4 in intracellular parasites as a function of time

(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 ($ROM4_{Nt}$).

(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*$_{S\text{-}A}$ 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.

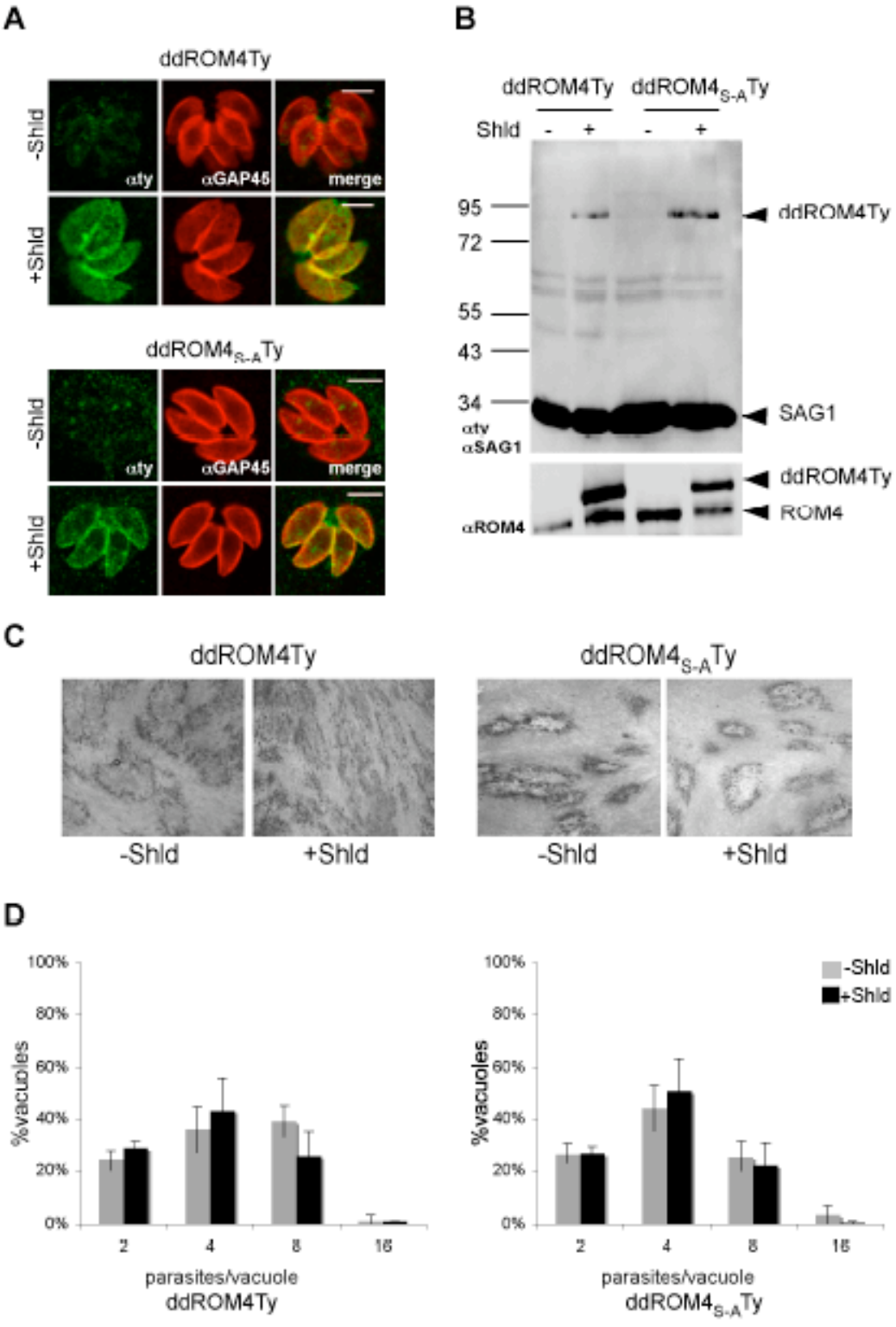

Figure S2. C-terminal epitope tagging of ddROM4$_{S\text{-}A}$ abrogates its dominant negative effect

(A) Expression of both ddROM4Ty and ddROM4$_{S\text{-}A}$Ty is efficiently stabilized by treatment with Shld-1 for 12 h as determined by indirect immunofluorescence assay

(IFA) using α-Ty antibodies (green). α-GAP45 antibodies (red) were used to stain the parasite. Scale bar, 5 μm.

(B) Western blot showing stable expression of ddROM4Ty and ddROM4$_{S\text{-}A}$Ty (α-Ty antibody, top panel) as compared to the expression of endogenous ROM4 (α-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$_{S\text{-}A}$Ty* parasites grown for 7 days ± Shld-1.

(D) Intracellular replication assays of *ddROM4Ty* or *ddROM4$_{S\text{-}A}$Ty* parasites. Parasites were grown 24 h ± Shld-1 prior to fixation. Data are represented as mean ± SD of three independent experiments.

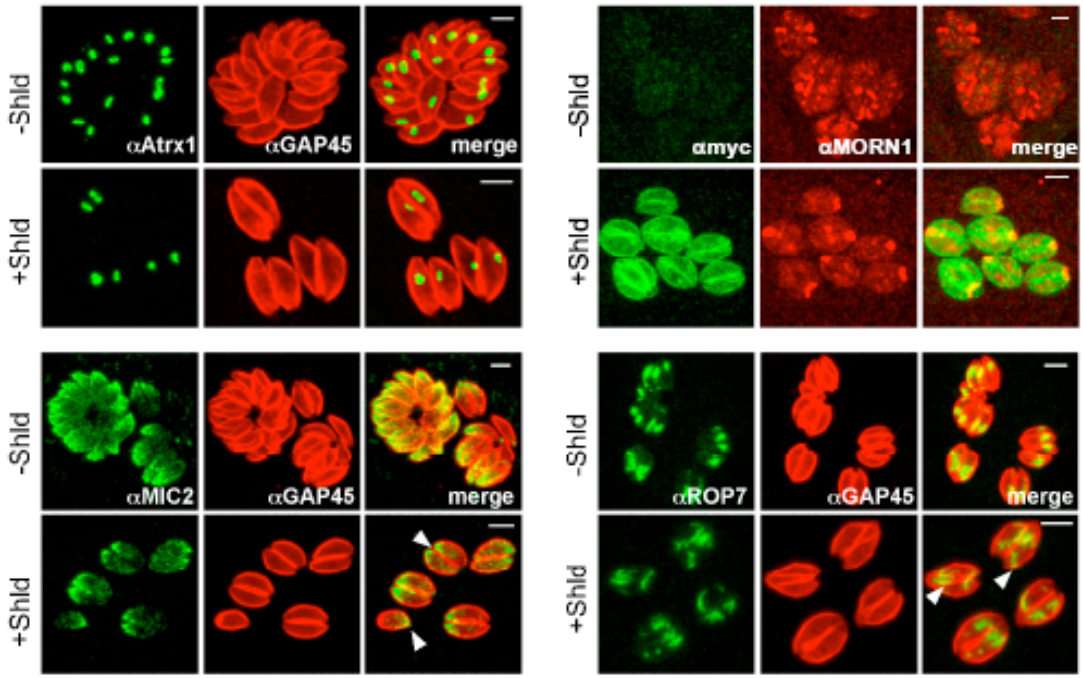

Figure S3. Assessment of the arrest in replication in *ddROM4*$_{S\text{-}A}$ parasites by IFA

Parasites expressing ddROM4$_{S\text{-}A}$ 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.

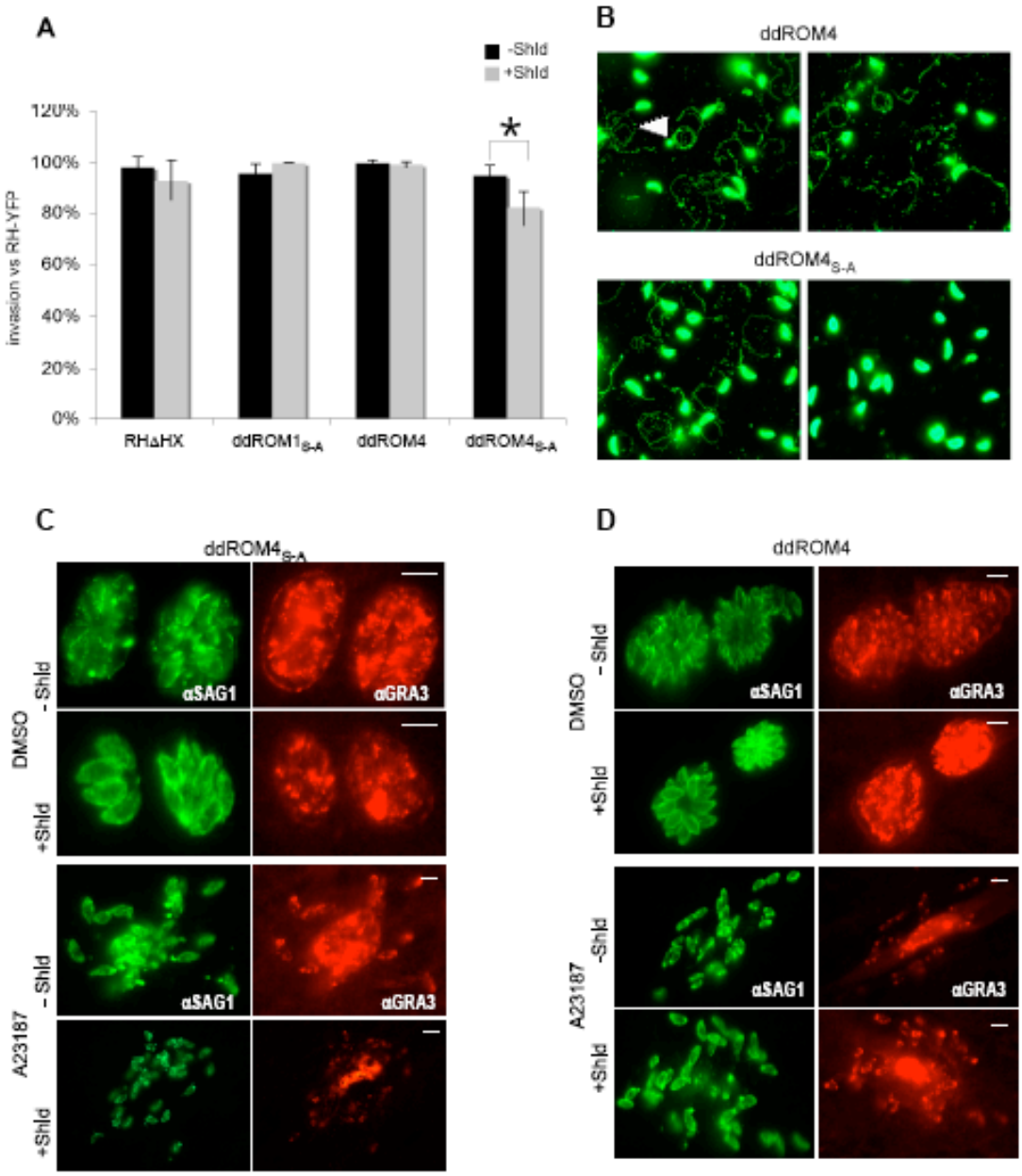

Figure S4 Shld-1 treatment of *ddROM4$_{S-A}$* parasites affects gliding, host cell invasion and egress

(A) Stabilization of ddROM4$_{S-A}$ expression in extracellular parasites treated for 6 h ± Shld-1 modestly affected host cell invasion, whereas expression of ddROM4 or ddROM1$_{S-A}$ 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.

(B) *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*$_{S\text{-}A}$ (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.

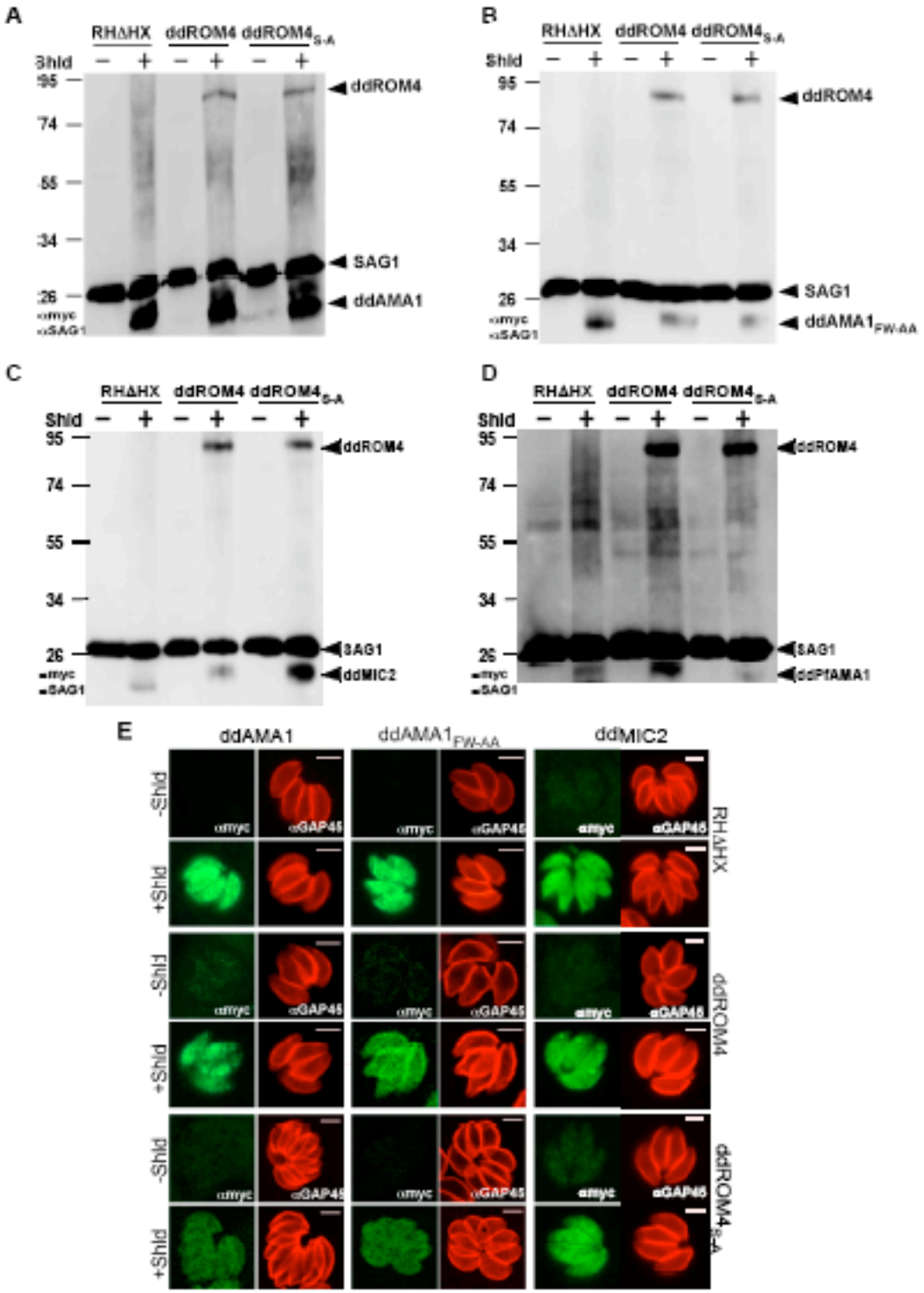

Figure S5 Expression of ddAMA1, ddAMA1$_{FW-AA}$, ddPfAMA1 and ddMIC2 in *RHΔHX*, *ddROM4* or *ddROM4$_{S-A}$* parasites is efficiently regulated by Shld-1

(A) Western-blot of *RHΔHX* (left), *ddROM4* (middle) or *ddROM4$_{S-A}$* (right) parasites stably expressing ddAMA1 as detected with α-myc. SAG1 was used as loading control.

(B) Western-blot of *RHΔHX* (left), *ddROM4* (middle) or $ddROM4_{S\text{-}A}$ (right) parasites stably expressing $ddAMA1_{FW\text{-}AA}$ as detected with α-myc. SAG1 was used as loading control.

(C) Western-blot of *RHΔHX*, *ddROM4* or $ddROM4_{S\text{-}A}$ parasites stably expressing ddMIC2 as detected with α-myc. SAG1 was used as loading control.

(D) Western-blot of *RHΔHX*, *ddROM4* or $ddROM4_{S\text{-}A}$ parasites stably expressing ddPfAMA1 as detected with α-myc. SAG1 was used as loading control.

(E) IFA of *RHΔHX*, *ddROM4* or $ddROM4_{S\text{-}A}$ parasites expressing ddAMA1, $ddAMA1_{FW\text{-}AA}$ 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.

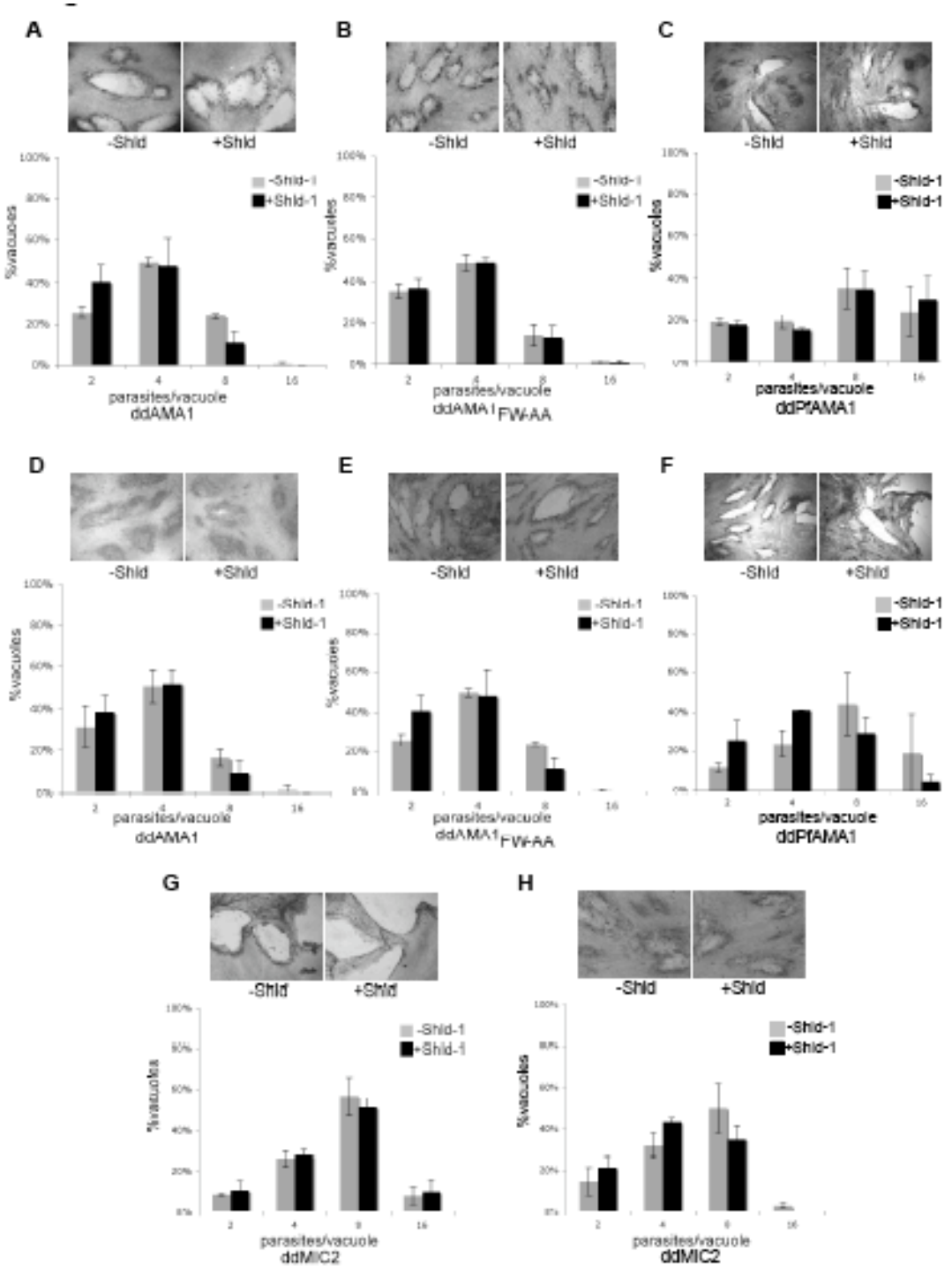

Figure S6 Plaque and intracellular growth assays of *RH* and *ddROM4* parasites expressing ddAMA1, ddAMA1$_{FW-AA}$, ddPfAMA1 or ddMIC2

Plaque assays and intracellular growth assays of *RHΔHX* parasites expressing ddAMA1 (A), ddAMA1$_{FW-AA}$ (B), ddPfAMA1 (C) or ddMIC2 (G). Plaque assays and intracellular growth assays of *ddROM4* parasites expressing ddAMA1 (D), ddAMA1$_{FW-AA}$ (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.

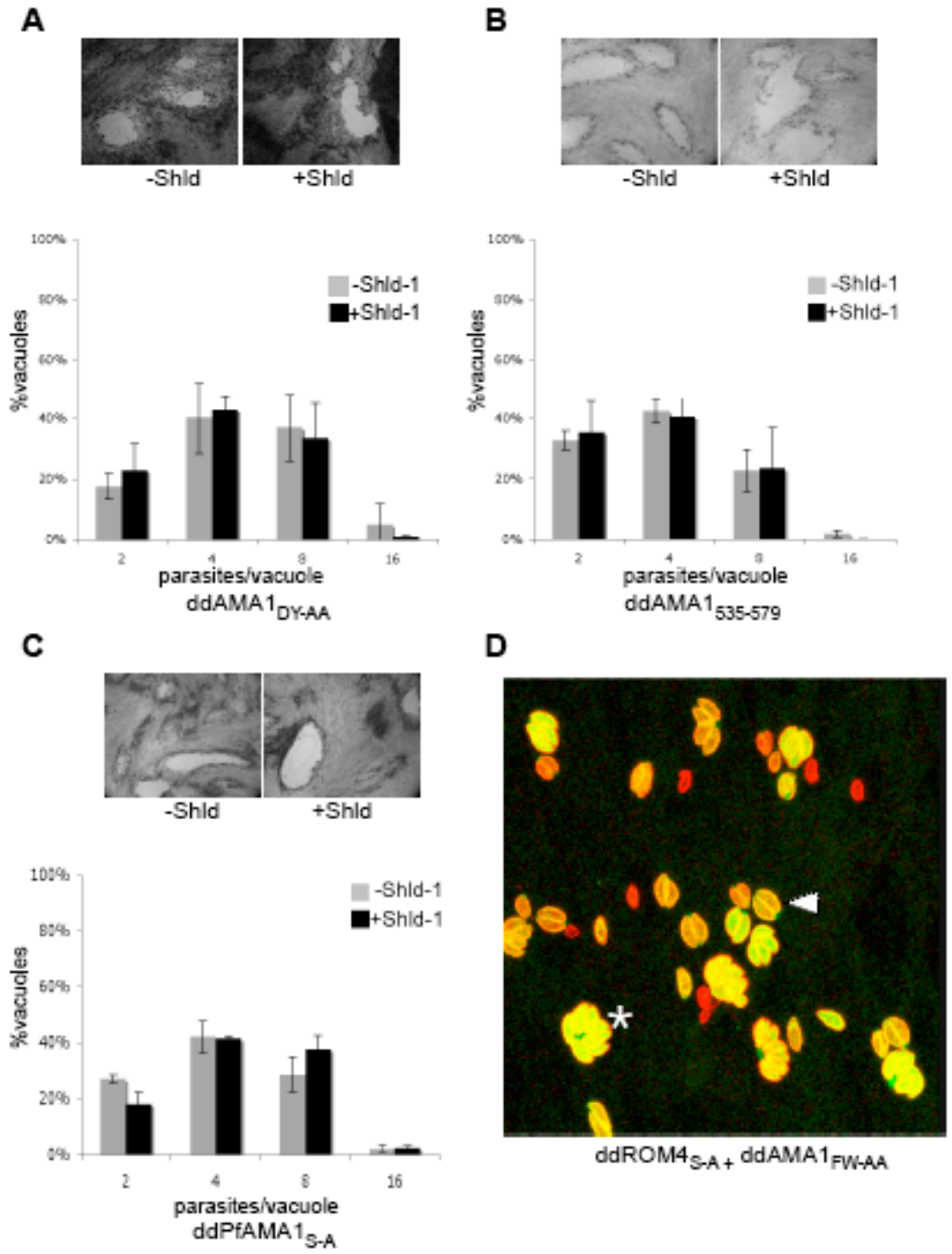

Figure S7 Plaque and intracellular growth assays of *ddROM4$_{S-A}$* parasites expressing ddAMA1$_{DY-AA}$, ddAMA1$_{535-570}$ or ddPfAMA1$_{S-A}$

Plaque and intracellular growth assays of *ddROM4$_{S-A}$* 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, respectively. Data are represented as mean ± SD as determined in three independent experiments.

(D) IFA of a mixed population of parasites expressing only ddROM4$_{S\text{-}A}$ (arrowhead) or ddROM4$_{S\text{-}A}$ and ddAMA1$_{FW\text{-}AA}$ (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$_{FW\text{-}AA}$ expression on parasite replication can be clearly observed.

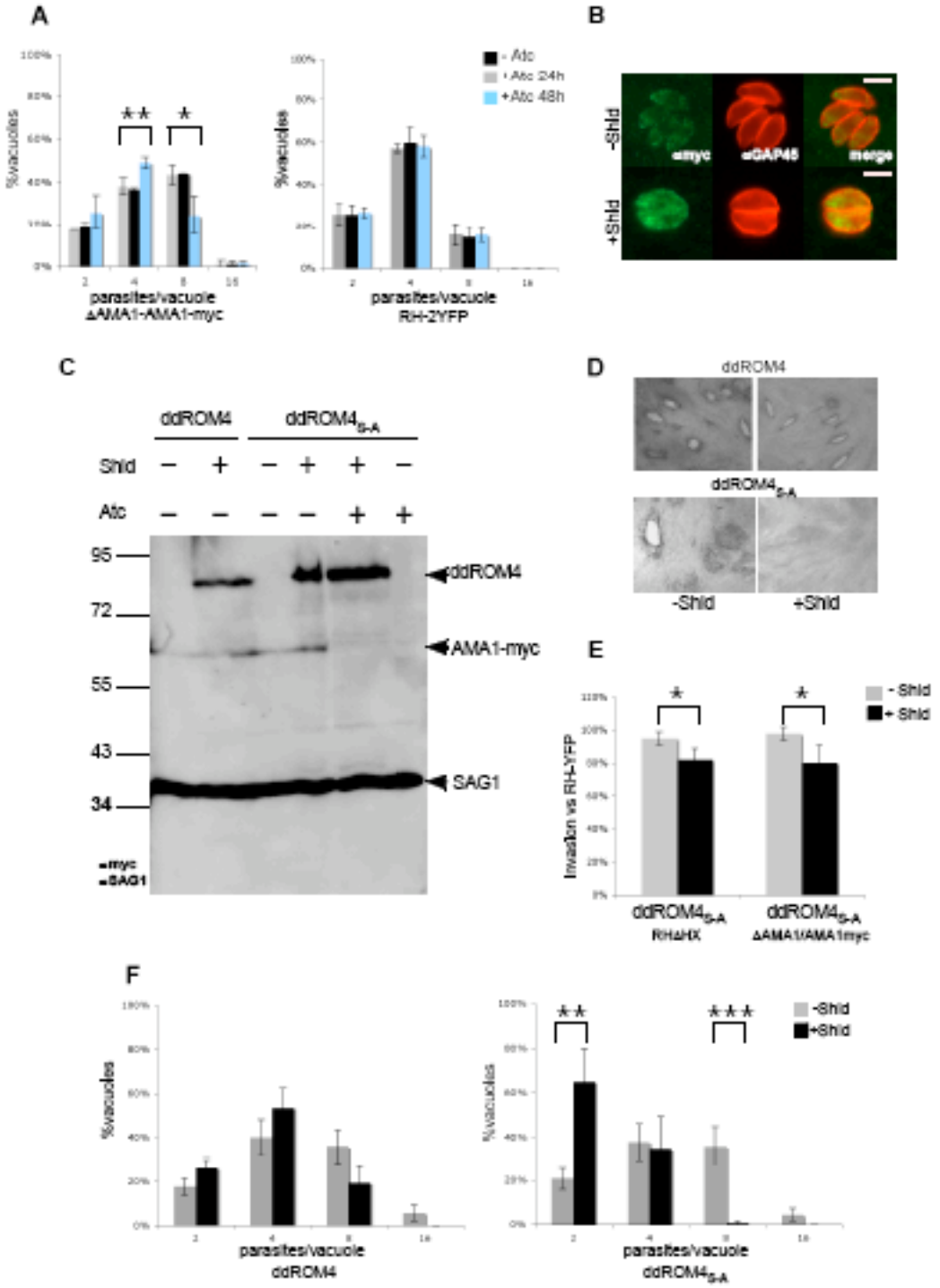

Figure S8 *ΔAMA1-AMA1myc* parasites are affected in cell division

(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

Primer name	**No.**	**Enz**	**Sequence**	**Resulting plasmid**
TUB8	2576	*KpnI*	GGTACCGGGCCCCCCCTCGACG	pT8ddmycHis
Myc-His	2577	*NsiI*	ATGCATATGGTGATGGTGGTGATGGTGGTGGGCCATGGCCAGGTCCTCC	pT8ddmycHis
ROM4	1483	*EcoRI*	CCGGAATTCCCTTTTTCGACAAAATGGTGTGGACTTCGGCCGTC	pT8ddmycROM4$_{S-A}$Ty
ROM4	1484	*NsiI*	GGCATGCATGCGGTTCAAGATAATACTGCGCATCC	pT8ddmycROM4$_{S-A}$Ty
ROM4	1516		CAGTCGGATCGGCTGGTTCCATGTATG	pT8ddmycROM4$_{S-A}$
ROM4	1517		CATACATGGAACCAGCCGATCCGACTG	pT8ddmycROM4$_{S-A}$
ROM1	1703		CTCTCAAAGTTGGAGCCGCTACGGCAGGCTTCGG	pT8ddmycROM1$_{S-A}$
ROM1	1704		CCGAAGCCTGCCGTAGCGGCTCCAACTTTGAGAG	pT8ddmycROM1$_{S-A}$
AMA1	2614	*NsiI*	ATGCATGGAGGCTGCTACTTCGCGAAGAG	pT8ddmycHisAMA1
AMA1	1080	*PacI*	CCGCAATTGTTAATTAACTAGTAATCCCCCTCGACCATAA	pT8ddmycHisAMA1
AMA1	1830		CATGCAAGAGGCTGAACCGTCGGCTGCGGATGAGGCAGAGGAGAAC	pT8ddmycHisAMA1$_{FW-AA}$
AMA1	1831		GTTCTCCTCTGCCTCATCCGCAGCCGACGGTTCAGCCTCTTGCATG	pT8ddmycHisAMA1$_{FW-AA}$
AMA1	3129	*NsiI*	CGATGCATCCAAGCGATCTCATGCAAGAGGCTG	pT8ddmycHis AMA1$_{535-570}$
AMA1	3131	*PacI*	CGTTAATTAACTACCAAAACGACGGTTCAGCCTCTTGC	pT8ddmycHis AMA1$_{504-549}$
MIC2	2945	*NsiI*	ATGCATGCAGCTGGAGGATTTGCATATAATTTTG	pT8ddmycHisMIC2
MIC2	2946	*PacI*	TTAATTAACTACTCCATCCACATATCACTATCGTC	pT8ddmycHisMIC2
PfAMA1	3140		CGAGGAGAAGCGCGCGGCGCACACGACGCCG	pT8ddmycHisPfAMA1$_{S-A}$
PfAMA1	3141		CGGCGTCGTGTGCGCCGCGCGCTTCTCCTCG	pT8ddmycHisPfAMA1$_{S-A}$

Table S2. Number of parasites per vacuole and percentage of *ddROM4*$_{S\text{-}A}$ parasites undergoing endodyogeny at different time points as determined by IFA and EM

Sample	Shld-1	% 1+2par/vac	%4+par/vac	% endodyogeny
18h	-	84	16	12[a]
	+	99	<1	5[a]
24h	-	32	68	21[a] 40[b]
	+	90	10	4[a] 10[b]
36h	-	[c]	[c]	[c]
	+	99	<1	5[a]

[a] determined by EM
[b] determined by IFA
[c] monolayer destroyed (no counts possible)

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

Shld-1	% Mitochondrion	% Micronemes	% Rhoptries	% Nucleus
-	< 5	< 5	< 5	<10
+	46	34	59	88

REFERENCES

S1. F. Plattner *et al.*, *Cell Host Microbe* **3**, 77 (2008).
S2. L. Sheiner, T. J. Dowse, D. Soldati-Favre, *Traffic* **9**, 665 (2008).
S3. C. Opitz *et al.*, *EMBO J* **21**, 1577 (2002).
S4. M. E. Rome, J. R. Beck, J. M. Turetzky, P. Webster, P. J. Bradley, *Infect Immun* **76**, 4865 (2008).
S5. A. E. DeRocher *et al.*, *Eukaryot Cell* **7**, 1518 (2008).
S6. J. R. Beck, Rodriguez-Fernandez, I.A., de Leon, J.C., Huynh, M., Carruthers, V.B., Morrissette, N.S., Bradley, P.J., *PLoS Pathog*, (2010).
S7. M. J. Gubbels *et al.*, *PLoS Pathog* **4**, e36 (2008).
S8. T. J. Dowse, J. C. Pascall, K. D. Brown, D. Soldati, *Int J Parasitol* **35**, 747 (2005).
S9. A. Herm-Gotz *et al.*, *Nat Methods* **4**, 1003 (2007).
S10. M. Meissner, D. Schluter, D. Soldati, *Science* **298**, 837 (2002).
S11. C. Hettmann *et al.*, *Mol Biol Cell* **11**, 1385 (2000).
S12. D. Soldati, J. C. Boothroyd, *Science* **260**, 349 (1993).
S13. R. G. Donald, D. Carter, B. Ullman, D. S. Roos, *J Biol Chem* **271**, 14010 (1996).
S14. L. Sheiner *et al.*, *Mol Microbiol*, (2010).
S15. J. S. Buguliskis, F. Brossier, J. Shuman, L. D. Sibley, *PLoS Pathog* **6**, e1000858. (2010).
S16. J. Mital, M. Meissner, D. Soldati, G. E. Ward, *Mol Biol Cell* **16**, 4341 (2005).
S17. M. H. Huynh, V. B. Carruthers, *PLoS Pathog* **2**, e84 (2006).
S18. C. G. Donahue, V. B. Carruthers, S. D. Gilk, G. E. Ward, *Mol Biochem Parasitol* **111**, 15 (2000).
S19. S. A. Howell *et al.*, *Mol Microbiol* **57**, 1342 (2005).