Apical membrane antigen 1 mediates apicomplexan parasite attachment but is dispensable for host cell invasion

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Apicomplexan parasites invade host cells by forming a ring-like junction with the cell surface and actively sliding through the junction inside an intracellular vacuole. Apical membrane antigen 1 is conserved in apicomplexans and a long-standing malaria vaccine candidate. It is considered to have multiple important roles during host cell penetration, primarily in structuring the junction by interacting with the rhoptry neck 2 protein and transducing the force generated by the parasite motor during internalization. Here, we generate Plasmodium sporozoites and merozoites and Toxoplasma tachyzoites lacking apical membrane antigen 1, and find that the latter two are impaired in host cell attachment but the three display normal host cell penetration through the junction. Therefore, apical membrane antigen 1, rather than an essential invasin, is a dispensable adhesin of apicomplexan zoites. These genetic data have implications on the use of apical membrane antigen 1 or the apical membrane antigen 1–rhoptry neck 2 interaction as targets of intervention strategies against malaria or other diseases caused by apicomplexans.
Most apicomplexans, including the agents of malaria (Plasmodium) and toxoplasmosis (Toxoplasma), are obligate intracellular parasites. Many invade host cells by a conserved mechanism involving the formation of a zone of tight attachment between the parasite apex and the host cell1, called tight junction (TJ). The current view is that the TJ is primarily a molecular bridge between the parasite sub-membrane actin-myosin motor and a stable and stationary anchor associated with the host cell surface/cortex, which allows parasite traction into a parasitophorous vacuole (PV) within the host cell2–4.

The transmembrane protein apical membrane antigen 1 (AMA1), arguably the most studied protein in the Apicomplexa phylum and a long-standing malaria vaccine candidate, is thought to shape the TJ on the parasite side5–11. The cytoplasmic tail of AMA1 was reported to bind aldolase8 in vitro, considered a signature of proteins that bind parasite actin and the motor12,13. The ectodomain of AMA1 tightly binds in parasite extracts to the rhoptry neck 2 (RON2) protein9,10, a protein secreted from the parasite rhoptries that specifically localizes at the TJ, where it inserts in the host cell membrane and is presumably linked to the cell cortical cytoskeleton via other RON proteins, like RON4. Moreover, antibodies or peptides that inhibit the AMA1–RON2 interaction drastically reduce host cell invasion by Plasmodium merozoites14–16 and Toxoplasma tachyzoites9,10. T. gondii or P. falciparum AMA1 bound to RON2 peptide were co-crystalized, revealing a conserved RON2 loop inserting deep into an AMA1 hydrophobic groove17,18. This reinforced the model of this interaction constituting the traction point used by the parasite to power the active internalization inside the PV19–21, and led to the proposal of developing broad-spectrum small-molecule inhibitors of apicomplexan invasion targeting the AMA1–RON2 interaction22–24. In addition, AMA1 has been reported to be involved in rhoptry secretion15,25 as well as for providing a signal initiating intracellular replication26.

Recently, P. berghei and T. gondii parasites in which AMA1 was silenced (AMA1 knockout, AMA1KO) were found to remain competent for shaping a TJ and invading host cells27, However, as AMA1KO parasites might still express residual levels of AMA1, these data were not considered as challenging any of the proposed roles of AMA1 in invasion18,28,29. In agreement with an essential role of AMA1 at some stage of the parasite invasion process, all attempts to inactivate AMA1 in both Plasmodium30,31 and Toxoplasma32 have failed so far.

Here, we report the inactivation of AMA1 in the tachyzoite of T. gondii, which invades virtually any cell type in the host, and in the merozoite and sporozoite stages of P. berghei, which invade erythrocytes and hepatocytes, respectively. AMA1 was deleted from the parasite genome by the diCre-loxP recombination approach in T. gondii and by direct homologous recombination in P. berghei. All three AMA1 knockout (AMA1KO) zootes are still capable of penetrating the respective host cell like the wild type (WT). Tachyzoites and merozoites, however, display a defect in host cell binding. These genetic data indicate that AMA1 and the RON proteins act separately during apicomplexan invasion, and that the AMA1–RON2 interaction does not have an essential role at the TJ.

Role of AMA1 in P. berghei merozoite infection of erythrocytes. To inactivate AMA1 in P. berghei, WT ANKA blood stages were transfected with a construct designed to replace endogenous AMA1 by pyrimethamine-resistance and green-fluorescence cassettes (Fig. 4a). Dedicated in vivo selection protocols with several days of drug pressure reproducibly generated mixtures of targeted green fluorescent protein (GFP+) AMA1− parasites, that is, AMA1KO, and non-targeted GFP− AMA1+ parasites, presumably spontaneous pyrimethamine-resistant mutants that typically emerge after long selection times. Southern blot analysis indicates the presence in the 20 reconstitution, recombinant parasites excise AMA1 (Fig. 1b) and express YFP. An excised clone, called TgAMA1KO, which does not produce AMA1 as shown by western blot (Fig. 1c) and immunofluorescence (Fig. 2a) analysis, was selected and maintained using routine culture procedures. When measuring parasite infectivity by plaque assay, the plaque size is 2 to 2.5 times smaller with TgAMA1KO than control parasites (Fig. 2a,b), a rather mild phenotype. Reintroduction of internally tagged AMA1FLAG in TgAMA1KO parasites fully restores overall growth, demonstrating the specificity of the observed phenotype (Fig. 2a).

We then investigated TgAMA1KO tachyzoite invasion of host cells in more detail. When measured by fluorescence microscopy, TgAMA1KO tachyzoite invasion efficiency is 30–40% that of the parental kub08:diCre strain (Fig. 3a). To investigate whether the pattern of TgAMA1KO tachyzoite invasion of host cells is normal or altered, TgAMA1KO tachyzoites invading human foreskin fibroblasts (HFFs) or normal rat kidney (NRK) fibroblasts were captured by confocal microscopy and analysed after three-dimensional reconstruction (Fig. 3b). Entering mutant zoites (n = 53) constantly display a typical RON4 circular staining around the parasite constriction site after cell permeabilization, indicating normal rhoptry secretion and TJ formation during invasion. In line with the normal gliding capacity of TgAMA1KO tachyzoites, micronemal protein 2 (MIC2)34, which is secreted like AMA1 from the microneme organelles, is normally exposed on the surface of invading TgAMA1KO tachyzoites (Fig. 3b). Moreover, video microscopy of invading TgAMA1KO zoites shows that successful invasion follows a one go and smooth process with similar kinetics as controls (Fig. 3c,d and Supplementary Movies 1–3), and in all cases (n = 20) a clear constriction site, suggestive of normal TJ, is observed. We conclude that in the tachyzoite AMA1 is not necessary for structuring a fully functional TJ, in which the RON proteins act independently of AMA1.

Next, we assessed tachyzoite adhesion to host cells. After 15 or 60 min incubation with live HFF cell monolayers, approximately two- to threefold fewer mutant versus control tachyzoites associate with host cells (total: extracellular attached and internalized combined), whereas three- to fourfold fewer mutants are located inside host cells, suggesting a primary defect of mutant zoites in host cell attachment (Fig. 3a). Importantly, about a third of the TgAMA1KO tachyzoite population adopts a distinct position relative to the host cell than controls, by binding only via the apical end rather than throughout their length (Fig. 3e), like previously observed for AMA1KO tachyzoites25. This confirms that AMA1 has an important role in tachyzoite adhesion to/positioning onto host cells before TJ formation, an event that favours, but is not required for, host cell invasion.
selected population of both the WT AMA1 locus and the expected allelic replacement (Fig. 4b). In agreement with this, immunofluorescence assays reveal erythrocytes infected by either GFP\(^+\) AMA1\(^-\) or GFP\(^-\) AMA1\(^+\) parasites (Fig. 4c).

The multiplication rate of AMA1\(^{KO}\) parasites, assessed by co-injection with control red fluorescent protein (RFP\(^+\)) parasites\(^{35}\) in mice and monitoring parasite multiplication by fluorescence-activated cell sorting (FACS), is \(~35\%\) that of...
Figure 2 | Infection of cell monolayers by TgAMA1KO tachyzoites. (a) AMA1 immunostaining in RH Δhxgprt, TgAMA1KO or TgAMA1FLAG multiplying inside host cells. Scale bar, 5 μm. Plaque assay with HFF cells after 6 days is shown on the right for each strain. Cells are stained with Giemsa. Scale bar, 0.5 mm. (b) Plaque area induced on HFF cells after 7 days infection with WT or TgAMA1KO tachyzoites. Mean area of ten plaques ± s.d. is depicted. *P-value < 0.0001 in unpaired Student’s t-test. (c) Pictures of HFF cell monolayers 20 h after co-infection with the ku80::diCre (non-fluorescent) and TgAMA1KO (green) strains. Scale bar, 10 μm. (d) Quantification of parasites per vacuole after cell infection. (e) Quantification of egressed vacuoles. Data show mean ± s.d. of three independent experiments. (f) Analysis of motility patterns. Data represent the mean ± s.d. of helical or circular trails associated with parasites counted in 30 fields of view. (b, d-f) The colour codes are shown in panel e. DAPI, 4',6-diamidino-2-phenylindole; KO, knockout.

Figure 3 | Host cell invasion by TgAMA1KO tachyzoites. (a) Red/green invasion assay. Attached and invaded parasites were numerated and normalized to total number of ku80::diCre. Data show mean of a representative assay out of three independent experiments. At least 400 tachyzoites were counted for each strain. (b) Invading parasites stained with antibodies against the surface (sMIC2) or total (tMIC2) micronemal protein MIC2, and with anti-RON4 antibodies to mark the TJ. Scale bars, 5 μm. The right panels (*) show processed three-dimensional (3D) images. Scale bars, 2 μm. Arrows indicate the direction of movement. (c) Penetration kinetics. Twenty independent events for each strain were recorded by time-lapse microscopy. (d) Time lapses of ku80::diCre (upper row) or TgAMA1KO (green, bottom rows) tachyzoites invading epithelial cells (cell types are indicated on the right). Numbers indicate seconds and the white arrows show the constriction sites, characteristic of TJ formation. Scale bars, 5 μm. (e) 3D reconstruction of the positioning of ku80::diCre or AMA1KO tachyzoites with labelled SAG-1 (green) attached to host cells expressing a fluorescent marker at the plasma membrane (red). Scale bars, 5 μm. The plot shows the angle of the parasite in relation to the mCherry-expressing host cell membrane measured for 50 tachyzoites of each strain. DIC, differential interference contrast.

RFP + parasites (Fig. 4d). As internalized AMA1KO parasites generate normal numbers of progeny merozoites after a normal developmental cycle (Fig. 4e), that is, AMA1 is not important for merozoite replication inside erythrocytes, the decreased multiplication rate of AMA1KO parasites reflects a defect in merozoite entry into erythrocytes.

We next characterized interactions between AMA1KO merozoites and erythrocytes using imaging flow cytometry (IFC),
which combines microscopy and flow cytometry and provides quantitative and functional information using imaging algorithms. Briefly (see Methods), after mixing mouse erythrocytes pre-stained with the lipid dye PKH26 with P. berghei GFP + merozoites36 collected from synchronized schizont cultures, parasites interacting with a host cell are identified as GFP signals in a gated PKH26 population (Fig. 5a, left panel), and internalized parasites are further recognized by co-localization of GFP with an increased PKH26 signal relative to the rest of the cell (Fig. 5b), a labelling suggestive of merozoites surrounded by a tight-fitting vacuole membrane37 (Fig. 5a, right panel). Using control GFP + merozoites incubated for 10 min with PKH26-stained erythrocytes before fixation, ~43.8% score as ‘associated’ with erythrocytes (EryA; Fig. 5a, left panel), whereas ~3.8% score as ‘internalized’ inside erythrocytes (EryI; Fig. 5a, right panel). Importantly, cytochalasin D, which prevents merozoite internalization but not attachment to erythrocytes38, does not significantly affect the EryA but drastically reduces the EryI population (Fig. 5a,c), which validates the EryI population algorithm. Using merozoites of the AMA1KO-containing population, ~8.6% of the GFP + AMA1KO merozoites score as EryA and ~0.48% as EryI (Fig. 5d). A similar reduction relative to control merozoites is obtained when samples are fixed after 3 min incubation (Fig. 5e), indicating a primary defect in adhesion of AMA1KO merozoites. Like AMA1KO tachyzoites, AMA1KO merozoites accumulate in the AMA1KO parasites propagated for extended times (up to 30 days) before IFC analysis, we next characterized AMA1KD merozoites generated by Flippase (Flp)/Flp Recombination Target (FRT)-mediated recombination27 immediately before IFC (Fig. 6a). In this approach, AMA1KD mosquito-stage sporozoites normally invade hepatocytes and transform into AMA1KD hepatic merozoites27. The latter cannot accumulate compensatory mutations before IFC, as they are generated in the absence of selection pressure and following a single invasion/multiplication cycle. We first analysed control hepatic merozoites.

![Image](https://www.nature.com/naturecommunications)
IFC analysis shows that ~44.3% and ~4.1% of control GFP\(^+\) hepatic merozoites score as Ery\(^A\) and Ery\(^I\), respectively, indicating that erythrocytic and hepatic merozoites bind and invade erythrocytes with similar efficiency in this assay. We then used AMA1\(^{KD}\) hepatic merozoites, composed of ~85% of excised AMA1\(^-\) parasites lacking any detectable AMA1 and ~15% of non-excised AMA1\(^+\) individuals used as internal controls (Fig. 6b). IFC analysis after AMA1 immunostaining (Fig. 7a) shows that ~48.8% and ~5.3% of AMA1\(^+\) controls score as Ery\(^A\) and Ery\(^I\), respectively, indicating that they behave like the
WT (Fig. 7b). In contrast, only ~3.3% of AMA1− merozoites score as EryA (Fig. 7b), that is, ~15-fold less than internal controls, demonstrating a major role of AMA1 in merozoite attachment. As expected, AMA1− merozoites also generate EryB events after 10 min (Figs. 6c and 7b) or 3 min incubation (Fig. 7c). Remarkably, EryB events are approximately fivefold less frequent in AMA1− than AMA1+ merozoites when normalized to input merozoites, but approximately threefold more frequent in AMA1− merozoites when normalized to attached parasites (Fig. 7b, P < 0.01, two-tailed t-test). Therefore, as with the Toxoplasma tachyzoite, AMA1 favours Plasmodium merozoite attachment to, but not internalization into, the host cell.

AMA1 has no role in P. berghei sporozoite infection of hepatocytes. Recent work using P. berghei AMA1 KD and RON4 KD sporozoites revealed strikingly distinct phenotypes, with essential and dispensable roles for RON4 and AMA1, respectively, during sporozoite invasion of hepatocytes27. To test AMA1 KD merozoite capacity to invade hepatocytes, populations of GFP+/AMA1 KD/ GFP− AMA1+ parasites were transferred to mosquitoes. The same ratio of GFP+ versus GFP− sporozoites is found in the blood fed to mosquitoes and in the mosquito salivary glands, indicating that AMA1 has no detectable effect on parasite development in the mosquitoes (Fig. 8a). The capacity of these salivary gland sporozoites to invade cultured hepatocytes was then tested. After sporozoite incubation with HepG2 cells in vitro, a similar proportion of AMA1 KD versus GFP− AMA1+ parasites is found in the input sporozoites and in hepatic schizonts developing inside HepG2 cells 60 h post infection (Fig. 8b). Likewise, in co-infection experiments of HepG2 cells with RFP+/AMA1+ as control, AMA1 KD sporozoites display similar invasive capacity as the control (Fig. 8c).

Finally, the infectivity of AMA1 KD sporozoites was tested in vivo. We found that intravenous injection into mice of as few as 500 AMA1 KD/AMA1+ sporozoites (Fig. 8d) or HepG2 cell-released hepatic merozoites (not shown) is sufficient to generate blood-stage parasite populations containing AMA1 KD parasites, demonstrating that parasites can complete a life cycle without producing AMA1. Moreover, injection into mice of only 50 AMA1 KD/AMA1+ infected erythrocytes is also sufficient to produce AMA1 KD-containing blood-stage populations (not shown). However, attempts of cloning AMA1 KD parasites were unsuccessful. This is likely due to the slower increase in parasitemia of AMA1 KD parasites, delaying the emergence of an AMA1 KD population that is eventually cleared by the mouse immune system before being detectable. Nonetheless, we cannot rule out the formal hypothesis that AMA1 KD parasites cannot be cloned because they require soluble AMA1 secreted from the AMA1+ counterparts. However, this hypothesis of AMA1 as an essential diffusible factor appears unlikely, as AMA1 KD growth is observed after co-injection of less than 50 blood stages and 500 sporozoites in the whole animal.

Discussion
We have inactivated AMA1 both in Toxoplasma and Plasmodium using dCre-loxP-mediated recombination and direct gene targeting, respectively, and found that AMA1-deficient T. gondii tachyzoites and P. berghei merozoites and sporozoites were still invasive and displayed a normal host cell penetration step. The most striking phenotype is that of AMA1 KD sporozoites, which showed no defect in hepatocyte invasion, confirming prior data obtained with AMA1 KD sporozoites that invaded hepatocytes even better than the WT27. This now demonstrates that AMA1 is dispensable for hepatocyte invasion and that, given the essential role of RON4 in the process27, the RON complex acts in an AMA1-independent manner. The lack of an invasion phenotype of AMA1 KD sporozoites strongly suggests that AMA1 is not involved in TJ function.

In contrast to sporozoites, AMA1-deficient merozoites and tachyzoites displayed an approximately three- to fivefold decrease in invasion compared to their GFP-labeled counterparts.
in overall invasion efficiency. However, like sporozoites, they penetrated host cells like the WT. They formed a normal constriction and a normal RON ring at the TJ, and tachyzoites were internalized at the normal average speed of \( B \) 20 s.

Moreover, quantitative IFC analysis indicated that AMA1 KD merozoites invaded erythrocytes better than controls when normalized to adherent merozoites, reminiscent of the increased infectivity of AMA1KD sporozoites.

The decrease in invasion efficiency of AMA1-deficient tachyzoites and merozoites, which showed no defect in host cell penetration, was associated with altered zoite adhesion to host cells. Fewer AMA1-deficient merozoites bound to erythrocytes in IFC experiments, including in 3° adhesion assays. Lack of AMA1 only modestly reduced the numbers of bound tachyzoites but affected their positioning onto cells, with AMA1-deficient tachyzoites more frequently adopting an upward position when compared with controls. AMA1 might thus be important in a pre-invasive zoite orientation step, as earlier proposed for merozoites39. A gradient of AMA1 on the zoite surface might create a gradient of interaction forces in a Velcro-like mechanism that might either apically reorient a zoite-expressing AMA1 mostly at its front end (merozoite) or flatten a zoite-expressing AMA1 all over its surface (tachyzoite). Why AMA1 has zoite-dependent contributions is unclear but might be related to zoite shape. A zoite-specific optimal positioning step, possibly involved in inducing rhoptry secretion, might be useful for the pear-shaped tachyzoites and merozoites and dispensable or even inhibitory for the naturally flattened sporozoites.

Therefore, genetic data indicate a model where AMA1 and the RON proteins have separate roles during apicomplexan invasion. AMA1 acts in a host cell-binding step that impacts the frequency but not quality of RON-dependent TJ formation, and the AMA1–RON2 interaction is not involved in the transduction of the force generated by the zoite motor during invasion. It can be argued that our data are still compatible with an essential function of
AMA1 at the TJ, if the residual invasion capacity of AMA1 mutants is ensured by an AMA1-like, functionally redundant protein. This hypothesis is highly unlikely for several reasons. First, the invasive AMA1 mutants displayed a normal entry phenotype including a fully functional TJ. This implies that any compensatory mechanism would need to be of optimal efficiency but expressed in only a subset of mutants (those that invade), a situation different from classical compensation by a suboptimal homolog that affects phenotype quality in all mutant parasites. Second, P. berghei AMA1KD sporozoites generated by Flp/FRT-mediated 3′-untranslated region (UTR) excision and T. gondii AMA1KD tachyzoites generated by Tet-mediated transcriptional repression were silenced immediately before phenotype analysis, thus precluding any selection of compensatory mechanism(s). Interestingly, AMA1KO T. gondii tachyzoites grown in continuous culture, which adapted by overexpressing the AMA1 homologue TgME49_300130 by ~15-fold (Fig. 1d), displayed a significantly milder adhesion phenotype, suggesting that the AMA1 homologue indeed compensated the adhesion defect of AMA1 mutants. The hypothesis of compensation at the TJ is also highly improbable in Plasmodium, which contains a single AMA1 gene. The parasite product most closely related to AMA1 is the transmembrane protein MAEBL40, which in P. berghei is only detected in oocyst sporozoites where it confers binding to the mosquito salivary glands but not invasion of hepatocytes11. Therefore, rather than AMA1-complementing TJ components, AMA1-related proteins in both Toxoplasma and Plasmodium appear to function in zoite adhesion, like AMA1.

One question raised by the model in which AMA1 and the RON proteins have dissociated functions is the role of the AMA1–RON2 interaction. The interaction is not essential but is important, being evolutionarily conserved. It might be required for processing/cleavage of surface AMA1 passing the TJ, perhaps allowing the disengagement of interaction of AMA1 with its host cell receptor and facilitating zoite sliding free into the PV. Interestingly, AMA1 undergoes a conformational change upon RON2 binding17, which could lead to loss of adhesive function or exposure of cleavage sites. This would reconcile the genetic data and the fact that antibodies or small molecules that inhibit the interaction can reduce zoite invasion9,10,14–16,18,24,42. The increased frequencies of Plasmodium merozoite (relative to adhesive parasites) and sporozoite invasion might also point to a modulatory/inhibitory role, possibly in preventing other interactions important for TJ formation. More work is needed to understand the exact contribution of the AMA1–RON2 interaction, which appears to impact AMA1 but not the TJ per se.

The demonstration of the dispensability of AMA1 in any step of host cell invasion by apicomplexan zoites does not question the potential efficacy of AMA1 as target of malaria prevention measures. A large body of work shows the efficacy of antibodies to AMA1 in blocking erythrocyte infection13–15, which might also reduce sporozoite invasion of hepatocytes16. Likewise, although

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**Figure 8 | In vitro and in vivo infectivity of AMA1KO sporozoites.** (a) AMA1 immunostaining of sporozoites dissected from mosquitoes fed with the pyrimethamine-resistant population of GFP+ AMA1KO/GFP+ AMA1+ parasites. Scale bar, 10 μm. (b) AMA1 immunostaining of hepatic schizonts 62 h after infection of HepG2 cells by sporozoites from a. Scale bar, 20 μm. (c) Quantification of exo-erythrocytic forms (EEFs, liver stages) developing in HepG2 cells infected with GFP+ (AMA1KO) or WT RFP+. Data show mean ± s.d. of EEFs per well as counted in 6 wells of a 96-well plate. (d) Diagnostic PCR of the pyrimethamine-resistant population that appears in the blood of mice after intravenous injection of sporozoites from a. Pa/b pair of primers is specific for the WT locus, and Pc/b is specific for the mutant (Mut) locus. Primers are illustrated in Fig. 4a. DAPI, 4′,6-diamidino-2-phenylindole.
the AMA1–RON2 interaction might not have any positive role in invasion, its inhibition by small molecules might still efficiently perturb invasion. Nonetheless, our finding that AMA1-less variants would be only partially impaired in adhesion while perturb invasion. Nonetheless, our finding that AMA1-less invasion, its inhibition by small molecules might still efficiently the AMA1–RON2 interaction might not have any positive role in

**Methods**

**Parasites.** *P. berghei* WT ANKA strain GFP fluorescent (GFP@HSP70)36, RFP fluorescent (L733)35, AMA1/Confl and AMA1/Indo were maintained in 3-week-old female Wistar rats or 3-week-old female Swiss mice. Mice or rats were infected with *P. berghei* parasites by intraperitoneal or intravenous injection. Parasitemia was followed daily by blood smears and FACS analysis. Anopheles stephensi (Sda500 strain) mosquitoes were reared at the Centre for Production and Infection of Anopheles (CEPIA) at the Pasteur Institute as described37. HepG2 cell for sporozoite preparation were cultured in Dulbecco's modified Eagle's medium (DMEM) or McCoy's 5A medium supplemented with 10% fetal calf serum, 2 mM glutamine and 25 μg/ml -1 gentamicin.

**All experiments using rodents were performed in accordance with the guidelines and regulations of the Pasteur Institute and are approved by the Ethical Committee for Animal Experimentation.**

**Cloning of DNA constructs.** To generate the plasmid pGFP-hDHFR-PbAMA1KO, the 3' UTR of PbAMA1 was amplified from *P. berghei* genomic DNA (gDNA) with primers 5'UTR PbAMA1 fw and cloned in sites NotI and SacI in a modified pUC18 plasmid containing GFP@HSP70 cassette36 in sites Sall and Sacl. Finally, 3'UTR hDHFR was removed from the previous plasmid and cloned in the latter in sites NolI and Sall.

To generate pSR770-loxP-Ama1-loxP-YFP-HXGPR, the TgAMA1 open reading frame (ORF) was amplified from T. gondii gDNA using primers TgAMA1 ORF fw/rv. In addition, 5'UTR and 3'UTR of ama1 were amplified from T. gondii gDNA using 5'UTR TgAMA1 fw/rv or 3'UTR TgAMA1 fw/rv, respectively. First, the 5'UTR of TgAMA1 was inserted upstream of pSR770-loxP-KillerRed-loxP-YFP-HXGPR plasmid in Apal restriction enzymes and transfected into ku80:-decrep recipient strain33. Parasites with stable integration were selected by the treatment with xanthine and mycophenolic acid. Integration by homologous recombination was confirmed by Southern blotting of AMA1IoxP strain was generated by replacement of the endogenous ama1 by floxed ama1 via homologous recombination. The targeting sequence pSR770-loxPAMA1-loxP-YFP-HXGPR was removed from plasmid by digestion with NolI and XhoI restriction enzymes and transfected into ku80:-decrep recipient strain33.

**Transfections and selection.** *P. berghei* genetic manipulation was performed as described39. *P. berghei* AMA1KO were generated by double homologous recombination to replace the endogenous ama1-coding sequence by a hDHFR cassette48 and a GFP fluorescence cassette. The targeting sequence with the two homologous regions flanking the selection cassettes was PCR amplified from plasmid pGFP-hDHFR-PbAMA1KO using primers 5'UTR PbAMA1 fw and 3'UTR PbAMA1 rv, and gel purified using the NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel) following kit instructions. After transfection of an enriched preparation of *P. berghei* ANKA schizonts and re-injection into mice, mutants were selected with constant treatment with pyrimethamine in drinking water until green fluorescent parasitemia was detected. Drugs were used as described49. The presence of AMA1KO was confirmed by PCR analysis with primers Pa/Pb, specific for the WT ama1 locus, and Pb/Pc, specific for integration at the ama1 locus, and by Southern blotting of total gDNA after digestion with the restriction enzymes MpiI and NdeI, with a probe hybridizing at the 5'UTR of ama1, amplified with primers 5'PbAMA1-probe fw/rv, to recognize the WT or the mutant loci with different sizes. For T. gondii genetic manipulation, ca 1 × 10⁸ of freshly lysed parasites were transfected with 60 μg linearized DNA by electroporation. Selection was performed with mycophenolic acid (12.5 mg ml⁻¹ in MeOH) and xanthine (20 mg ml⁻¹ in 1 M KOH), or phleomycin (50 μg ml⁻¹ in water).

**Southern and western blotting.** gDNA from *P. berghei* and *T. gondii* was used as a PCR template and for Southern blotting was extracted using Qiagen dneasy blood and tissue kit according to manufacturer’s protocol. For Southern blotting of *P. berghei* gDNA, samples were digested with MfeI and NdeI restriction enzymes overnight, precipitated with ethanol, washed and separated in agarose gel. The gel was transferred to a Hybond-XL membrane (GE-Healthcare) and blotting was performed using the DIG easy Hyb kit and DIG wash and block buffer kit from Roche according to manufacturer’s protocol. The probe was amplified with primers 5'PbAMA1-probe fw/rv using the DIG Probe Synthesis kit from Roche. Tachyzoite western blot samples were obtained by spinning down extracellular parasites and incubating with RIPA buffer (50 mM Tris-HCl pH 8; 150 mM NaCl; 1% Triton X-100; 0.5% sodium deoxycholate; 0.1% SDS; 1 mM EDTA) for 20 min on ice. Unless indicated otherwise 10⁷ parasites were loaded onto a SDS acrylamide gel for electrophoresis.

**Table 1 | Primers used in this study.**

| Primer | 5'-3'  |
|--------|--------|
| 3'UTR PbAMA1 fw | GGCGTCGAGAAATAGCGCCTTGGTTAATA |
| 3'UTR PbAMA1 rv | GGCGTCGACGGACGATTTAGGAAAGACCT |
| 5'UTR PbAMA1 fw | GGGAATTCATATATCAGTATAGC |
| 5'UTR PbAMA1 rv | GGGAAGCTCTTATATAGCTT |
| 5'PbAMA1-probe rv | CTATTTCACAGTACCAT TT |
| 5'PbAMA1-probe fw | CAATTTACATCAATATTTTAA |
| Pa (PbAMA1 cds fw) | GAATATGATGTTGAAATAAAG |
| Pb (PbAMA1 3'UTR rv) | TTTATTTAATAGCCCATATAAT |
| Pc (hDHFR fw) | GTTGAGCACTTAAAAGCACA |
| P3 (5'UTR TgAMA1 fw) | GGAGGGCCCATGAGTATGAGTACGAGACTGAG |
| 5'UTR TgAMA1 rv | GGAGGGCCCGAGACCACAACTGATGTAACG |
| 3'UTR TgAMA1 fw | GGAGGCTCCTGCGGCTAGTACCAATCCCTTATCAC |
| 3'UTR TgAMA1 rv | GAGGATCTGTGGAGAAGAACGTTGACCAGT |
| TgAMA1 ORF fw | GTAATTTAATAGATACATGGCTACACAATAGT |
| P2' (TgAMA1 ORF rv) | GAGCTCAAGCTTGATTCAGATG |
| P1 (5'UTR TgAMA1out fw) | CCGAGAATCTATGAGTCGAGTAC |
| P1’ (5'PbAMA1 fw) | TGGACGAGCTGCAAGGTG |
| P2 (TgAMA1int fw) | GTGAGCAGAGATGAGTACGGAAGG |
| P3’ (YFP rv) | TGACATTACCTGTCAGAGC |

Fw, forward; ORF, open reading frame; rv, reverse; UTR, untranslated region.
gel and immunoblot was performed as previously described. Briefly, proteins were transferred onto a nitrocellulose membrane, after blocking the membranes were incubated with primary antibody for 1 h (mouse anti-AMA1: 1:1,000; rabbit anti-aldolase: 1:10,000) followed by incubation with horseradish peroxidase-labelled secondary antibodies (1:50,000; Jackson ImmunoResearch) for 2 h.

Quantitative PCR. RNA from freshly egressed parasites was purified using Trizol followed by chloroform extraction. For cDNA synthesis, 2.5 μg of total RNA were retrotranscribed using the SuperScript VIGO (Invitrogen, Life Technology). Quantitative real-time PCR was performed on a LightCycler 480 (Roche) using the LightCycler 480 SYBR Green I Master Mix (Roche). PCR primers were designed to amplify a 100-bp target gene fragment: AMA1 Fw: 5′-TGGAAGAAACAAGGATGGGTTCTCT-3′; AMA1 Rv: 5′-CAGTAGTGAAGGACCAGGCCGC-3′; TgME9_300130 Fw: 5′-CCAGCAAGCACTGCCCCGTG-3′; TgME9_300130 Rv: 5′-AAACCCCTCGGCCTGTCCT-3′. cDNA levels were normalized to 2-tubulin levels measured with primers: Fw: 5′-GCAATGTACGCAACGACCT-3′; Rv: 5′-ACATACAGTGCCAGAGGC-3′. Experiments were performed four times with two different RNA preparations.

Immunofluorescence. For P. berghei merozoites, sporozoites and infected HepG2 cells immunofluorescence, samples were fixed with 4% paraformaldehyde, 0.0075% glutaraldehyde in PBS for 1 h, permeabilized with 0.1% Triton X-100 in PBS, blocked with BSA 3% in PBS, and stained with primary rabbit polyclonal antibodies to the P. berghei AMA1 peptide CRASHTTPVMKQPY (Eurogentec, 1:500 dilution), or primary polyclonal antibodies to the P. berghei RON2 peptide KKLGLKREIKIV3GFLFKRKG (Thermo Scientific, 1:500 dilution), followed by secondary Alexa-Fluor-conjugated antibodies (Molecular Probes, 1:500 dilution). Images were acquired using an Axiovert II fluorescence microscope (Zeiss) or the ImageStreamX from AMNIS.

For T. gondii immunofluorescence analysis, infected HFF monolayers grown on coverslips were fixed in 4% paraformaldehyde for 20 min at room temperature, followed by permeabilization (0.2% Triton X-100 in PBS) and blocking (2% BSA and 0.2% BSA to 0.2% Triton X-100 in PBS). The staining was performed using primary antibody (mouse anti-AMA1, 1:1,000; mouse anti-SAG1, 1:1,000; rabbit anti-MIC2, 1:500; rabbit anti-IMC1, 1:500; rabbit anti-GAP45 1:1,000) followed by secondary Alexa-Fluor-conjugated antibodies (Molecular Probes, 1:3,000). Images were acquired with a camera under Delta Vision Core or confocal Nikon Ti eclipse microscopes (z-stacks of 0.2–0.3 μm, ×100 immersion objective), deconvolved using SoftWoRx Suite 2.0 (Applied Precision, GE) when needed and further processed using ImageJ 1.34r and Photoshop (Adobe Systems) software.

Production of merozoites and ImageStream analysis. Erthyrocytic merozoites were obtained by culturing infected rat or mouse blood for 16 h, at 37°C, 5% CO2 and 10% O2, under shaking (90 r.p.m.), in RPMI 1,640 medium (Gibco) supplemented with 20% fetal calf serum and 50 μg/ml neomycin. Mature schizonts were separated in a Nycodenz gradient and merozoites were isolated by filtration of coverslips in a 6-well plate, transfected with 1 μg of plasmid-encoding mCherry in the pDisplay Vector (Invitrogen) and used 20 h later for a 5-min invasion assay. Cells were fixed in PBS-4% PFA (20 min, room temperature) and stained with anti-SAG-1 antibodies followed by Alexa-Fluor anti-mouse antibodies to label extracellular parasites. Samples were scanned on the confocal Nikon Ti Eclipse microscope and images were captured and analysed with Metamorph software (Universal VIEWER application). For confocal microscopy, a measure the longitudinal axis, whereas the cell surface contacting the tachyzoite centres of mass was affected to all the isosurfaces. The plane of the cell was reconstructed and angle values between the longer axis of the parasite and the host cell plane were generated by Metamorph.

T. gondii replication assay. 1×106 kbd40-diCre or TgAMA1FLAG or 5×105 TgAMA1KO were inoculated onto a confluent monolayer of HFF grown on coverslips (24-well plate) and incubated in normal growth conditions. One hour post inoculation, coverslips were washed in PBS to remove extracellular parasites and thus synchronize the cell cycle. Cells were further grown in normal growth conditions until as indicated, fixed and immunostained. The number of parasites per vacuole was determined for 100 vacuoles.

T. gondii plaque assay. 200 RH Ahxgpt or TgAMA1FLAG or parasites or 1,000 TgAMA1KO parasites were added onto a confluent monolayer of HFF cells of a six-well plate. After incubating for 6 h, the HFF monolayer was washed in PBS and fixed in ice-cold methanol for 20 min. Afterwards, the HFF cells were stained with Giemsa. The area of ten plaques was assessed using Image J 1.34r software.

T. gondii egress assay. 4×105 parasites were grown in HFF monolayers on coverslips for 36 h. Media were exchanged for pre-warmed, serum-free DMEM supplemented with calcium ions (AT31) and polybrene (12 μg/ml). Forty hours after infection with parasites were scored in 30 fields.

T. gondii motility assay. Freshly egressed tachyzoites were allowed to glide for 30 min on glass coverslips coated with 50 μg/ml heparin in PBS. Parasites and tissue culture medium were stained with anti-P30 antibodies and visualized with an inverted laser scanning microscope (Eclipse Ti, Nikon). Images were analysed using Metamorph and ImageJ software. Numbers of helical and circular trails associated with parasites were scored in 30 fields.

T. gondii video microscopy. Time-lapse video microscopy was conducted with the DeltaVision® Core microscope using a ×40 immersion lens. Freshly lysed RH Ahxgpt, kbd40-diCre or TgAMA1KO were added onto HFF. HeLa or U373 monolayer in glass dishes (ibidi; µ-Dish35 mm; 10 μl). Forty hours post inoculation, invasion of freshly egressed parasites was observed. Normal growth conditions were maintained throughout the experiment (37°C; 5% CO2). Images were recorded at one frame per second. Further image processing was performed using ImageJ 1.34r software and with Photoshop (Adobe Systems).

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Author contributions
D.Y.B. and S.T. designed and performed the experiments with *Plasmodium*, N.A., V.L., J.A.W. and I.T. designed and performed the experiments with *Toxoplasma*. I.T., M.M. and R.M. conceived this study and designed experiments. D.Y.B., N.A., V.L., I.T., M.M. and R.M. wrote the paper.

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