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

Joana M. Santos,[1,3] David J. P. Ferguson,[2] Michael J. Blackman,[3] Dominique Soldati-Favre[1]*

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.

Host cell invasion by *Toxoplasma* and *Plasmodium* involves discharge of secretory organelles called micronemes (*1*) and rhoptries. Apical membrane antigen 1 (AMA1), a microneme protein, is crucial for invasion (*2*) and is part of the moving junction complex formed during invasion (*3*). In *Toxoplasma gondii*, AMA1 and microneme protein–2 (MIC2), MIC6, MIC8, and MIC12 are cleaved during invasion within their transmembrane domain (TMD) by a rhomboid activity (*4*–*7*), releasing them from the parasite surface. In *Plasmodium*, the majority of AMA1 shedding is mediated by a subtilisin protease (*6*, *8*, *9*), but cleavage by a rhomboid protease also occurs (*6*). The role of AMA1 shedding is unknown. *Toxoplasma* encodes six rhomboids (*10*), two of which have been functionally dissected: ROM1 is expressed in the micronemes but does not play a crucial role in invasion (*11*, *12*); ROM4 localizes to the plasma membrane (*13*, *14*) and cleaves MIC2, AMA1, and MIC8 (*15*). *Plasmodium falciparum* ROM4 sheds the micronemal erythrocyte-binding protein EBA175 (*16*) and possibly other adhesins (*17*). After invasion, the parasite initiates replication within a parasitophorous vacuole. *Toxoplasma* tachyzoites divide by endodyogeny, in which repeated cycles of replication produce numerous new parasites equipped for egress and invasion. In contrast, *Plasmodium* replicates by schizogony, in which a multinucleated syncytium (schizont) is formed that undergoes cytokinesis only after completion of nuclear division. The signals governing initiation of replication are unknown.

Rhomboids are broad–substrate-specificity serine proteases that recognize helix-destabilizing residues within the substrate's TMD (*18*). We reasoned that expression of a *T. gondii* ROM4 mutant, able to bind the substrate but unable to cleave it, would sequester the substrate from the endogenous protease and behave as dominant negative. To regulate expression of the protease, we used the FK506 binding protein destabilization domain (dd) system in which fusion proteins are degraded unless a ligand, Shld-1, is added (*19*). We expressed either a control wild-type (WT) construct (ddROM4) or a mutant form, in which the catalytic Ser^{409} was substituted with an Ala residue ($ddROM4_{S\text{-}A}$) (*20*). Similar mutations ablate rhomboid activity (*13*, *14*, *17*). To validate the system, a ROM1 mutant ($ddROM1_{S\text{-}A}$) was also generated. The transgenic proteins were correctly localized and expressed in a Shld-1–dependent manner (Fig. 1, A and B). Expression of ddROM4 was detectable 5 min after Shld-1 treatment, but levels similar to those of the endogenous protein and correct trafficking were only reached by 480 or 180 min, respectively (fig. S1, A and B).

Plaque assays reflect multiple lytic cycles, including invasion, replication, egress, and invasion of neighboring cells. Parasites expressing ddROM4 or yellow fluorescent protein (RH-2YFP) formed plaques of similar size with or without Shld-1, indicating that overexpression of ROM4 was not detrimental. The $ddROM1_{S\text{-}A}$ parasites formed slightly smaller plaques in the presence of Shld-1, suggesting a modest growth defect. The $ddROM4_{S\text{-}A}$ parasites produced no plaques in the presence of Shld-1 (Fig. 1C), indicating that its expression exerts a dominant negative effect. Closer examination of the $ddROM4_{S\text{-}A}$ phenotype revealed an unanticipated defect in replication. Quantification of the number of parasites per vacuole 24 hours postinvasion indicated that the *RH-2YFP* and $ddROM1_{S\text{-}A}$ parasites replicated at a similar rate regardless of the presence of Shld-1, whereas the *ddROM4* parasites grew slightly better. In contrast, the $ddROM4_{S\text{-}A}$ line grew normally without Shld-1 but was severely impaired in the presence of Shld-1 (Fig. 1D and fig. S1C). Modification of the extreme C terminus of rhomboids interferes with activity (*13*, *14*, *16*), and inclusion of a C-terminal Ty-1 epitope tag abrogated the deleterious effect of $ddROM4_{S\text{-}A}$ (fig. S2), supporting the notion that stabilization of $ddROM4_{S\text{-}A}$ produces a dominant-negative effect.

Delivery of organelles into daughter cells during replication occurs in a highly coordinated fashion, starting with the centriole and Golgi, followed by the apicoplast, the nucleus, and endoplasmic reticulum, and finishing with the mitochondrion and de novo synthesis of the micronemes and rhoptries (*21*). To define the point of cell-cycle arrest after $ddROM4_{S\text{-}A}$ stabilization, we scrutinized the integrity and inheritance of various organelles. Though the inner membrane complex and apicoplast appeared normal, the mitochondrion, micronemes, rhoptries, and nuclei were defective in arrested parasites (Fig. 2, fig. S3, and tables S2 and S3) in a phenotype characteristic of an arrest late in the cell cycle at the S phase (*22*). $ddROM4_{S\text{-}A}$ stabilization also reduced the amount of vacuoles containing developing daughter cells (Fig. 2B and table S2).

Because ROM4 plays an important though nonessential role during invasion (*15*), we investigated the effect of $ddROM4_{S\text{-}A}$ stabilization on motility and invasion. As prolonged Shld-1 treatment of intracellular parasites prevented egress as a direct result of the block in replication, we stabilized $ddROM4_{S\text{-}A}$ in nondividing extracellular parasites. Consistent with (*15*), there was a modest defect in invasion and gliding (fig. S4).

To determine whether the impairment in cell division after $ddROM4_{S\text{-}A}$ stabilization was dependent on an invasion-related event, we performed pulse-chase assays. Although $ddROM4_{S\text{-}A}$ parasites treated with Shld-1 for 12 hours before egress and for 6 hours postinvasion recovered and underwent normal cell division (Fig. 3A), parasites treated at 6 hours postinvasion were impaired (Fig. 3B). Thus, the defect is reversible and independent of invasion, resulting from the noncleavage of one or more substrates at the intracellular parasite surface, whose processing is required to trigger or maintain replication.

All *T. gondii* rhomboid substrates characterized to date are micronemal proteins. Among these, AMA1 is unique in that it functions exclusively during invasion (*2*), it is detected on invading parasites (*3*), and its C-terminal tail is detectable in the newly invaded (*6*, *7*, *9*) parasites. We hypothesized that the AMA1 cytosolic tail generated by ROM4 cleavage during invasion triggers parasite replication and that stimulation of each replicative cycle is engendered by further cleavage of AMA1 or another substrate. To test this hypothesis, we determined whether the block in division could be reverted by expressing the *T. gondii* (ddAMA1) or *P. falciparum* (ddPfAMA1) AMA1 tail in either the WT form or mutated in residues important for the AMA1 invasion-related function (*23*–*25*). We substituted with Ala either the conserved Phe and Trp (*23*) (FW) (*26*) residues in TgAMA1 ($ddAMA1_{FW\text{-}AA}$) or the Ser residue phosphorylated in PfAMA1 (*25*)

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(ddPfAMA1$_{S-A}$) (Fig. 4A). Expression of all transgenes was Shld-1–regulated (fig. S5) and did not affect the growth of WT or *ddROM4* parasites (fig. S6), but it was able to rescue the replication phenotype of *ddROM4$_{S-A}$* parasites (Fig. 4, B and C, and fig. S7), indicating independent, dual functions for AMA1 in replication and invasion. To identify residues involved in the replication function of AMA1, we tested ddAMA1 constructs carrying an Ala replacement at the conserved C-terminal Tyr residue (ddAMA1$_{DY-AA}$) or with the most N-terminal region (ddAMA1$_{535-570}$) or the 20 most C-terminal residues (ddAMA1$_{504-549}$) deleted (Fig. 4A). None of the mutants impaired function (Fig. 4, B and C, and fig. S7), suggesting that the conserved central region PSDLMQEAEPS is important for function. To assess the specificity of these results, we expressed the MIC2 cleavage product (ddMIC2) and verified that it did not affect parasite growth (fig. S6) or complement the ddROM4$_{S-A}$-mediated arrest (Fig. 4, B and C).

Two previous studies found no evidence for a role of ROM4 or AMA1 in replication (*2*, *15*). Reexamination of the phenotype of parasites conditionally depleted for *AMA1* (*2*) found that they were modestly affected in division (fig. S8), con-

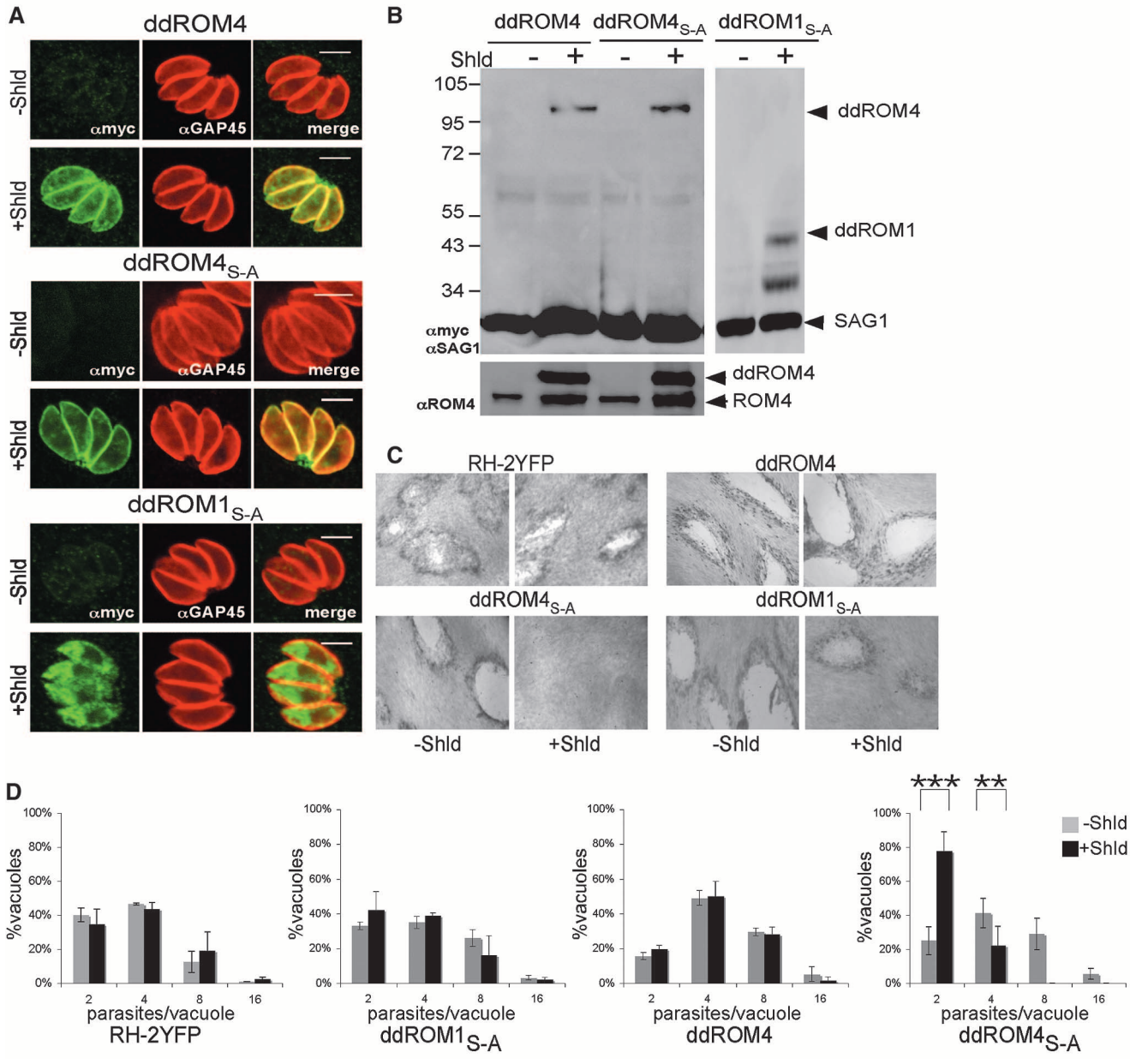

Fig. 1. Expression of ddROM4$_{S-A}$ severely impairs intracellular growth. Stabilization of ddROM4, ddROM4$_{S-A}$, and ddROM1$_{S-A}$ expression on parasites treated 12 hours ± Shld-1, as shown by indirect immunofluorescence assay [(**A**), green] or Western-blot [(**B**), top panels] with α-myc antibodies. The additional lower form of ddROM1$_{S-A}$ (B) probably corresponds to a degradation product or otherwise modified form (*13*). Gliding-associated protein 45 (α-GAP45) labels the inner-membrane complex [(A), red]. Scale bars in (A): 5 μm. ROM4 N-terminal (α-ROM4) antibodies were used to compare the expression level of ddROM4/ddROM4$_{S-A}$ to endogenous ROM4 [(B), bottom panel]. The surface marker surface antigen 1 (αSAG1) served as loading control [(B), top panels]. (**C**) Plaque assays of *RH-2YFP*, *ddROM4*, *ddROM4$_{S-A}$*, and *ddROM1$_{S-A}$* parasites grown 7 days ± Shld-1. The assays were performed simultaneously for the same parasite strain ± Shld-1. (**D**) Replication profile of *RH-2FP*, *ddROM1$_{S-A}$*, *ddROM4*, and *ddROM4$_{S-A}$*, parasites pretreated 12 hours ± Shld-1 before host cell egress and for the time of the assay in a total of 36 hours. We counted the number of parasites per vacuole 24 hours after host cell inoculation. Asterisks indicate statistically significant results (**P = 0.004; ***P = 0.0005), as determined by the Student's t test. Data are represented as mean ± SD (error bars) of four independent experiments.

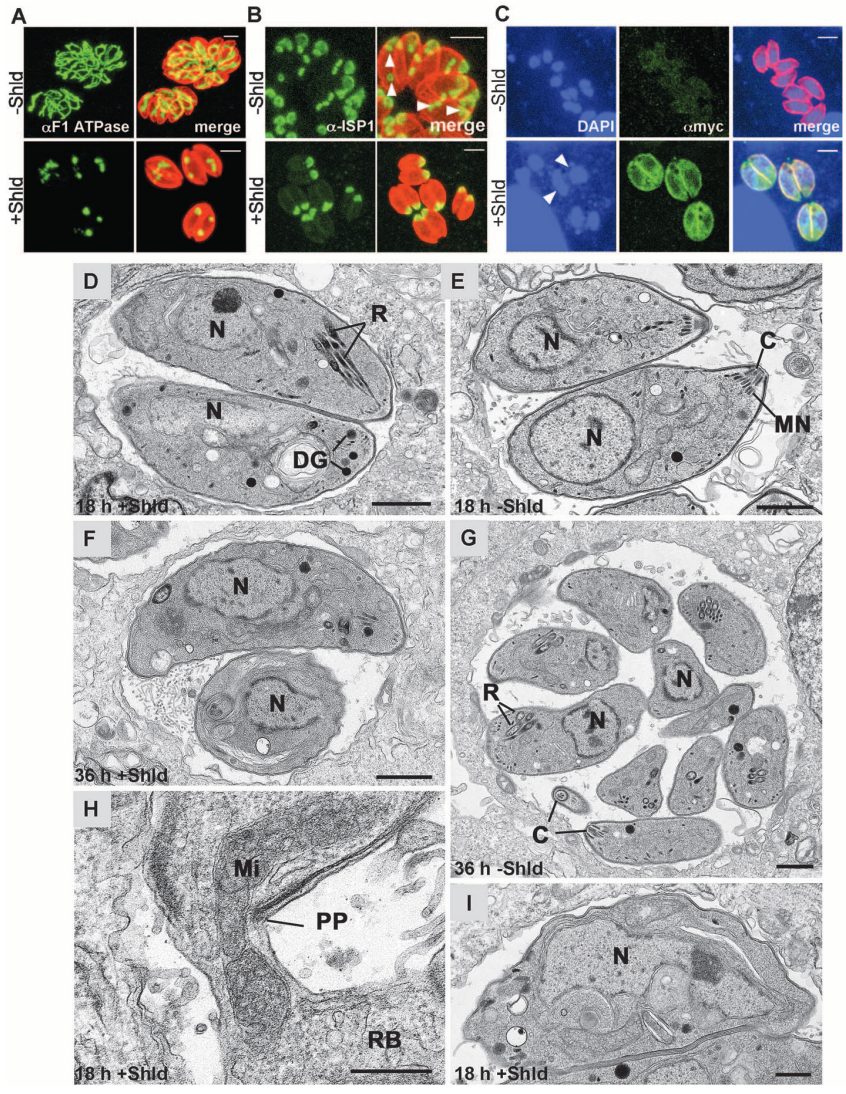

Fig. 2. *ddROM4$_{S\text{-}A}$* parasites arrest late in the cell cycle. Indirect immunofluorescence assay of *ddROM4$_{S\text{-}A}$* parasites after 24 hours ± Shld-1 showing the mitochondrion (α-F1 adenosine triphosphatase β subunit, green) broken down (**A**); replication arrested after a single round of division, as determined by staining of the nascent apical cones of the mother and daughter parasites (α-ISP1, green) only in nontreated parasites [(**B**), arrowheads]; and defective karyocytokinesis with the nuclei enlarged and uncondensed [(**C**), arrowheads]. In red, parasites are labeled with α-GAP45. Longitudinal section through two daughters cells after 18 hours plus (**D**) or minus (**E**) Shld-1 and after 36 hours plus Shld-1 (**F**). (**G**) Section through a nontreated vacuole at 36 hours showing three cycles of endodyogeny. (**H**) Detail of a vacuole 18 hours plus Shld-1, showing the mitochondrion running between the posterior pole and the residual body, which is left after the budding of daughter cells from the mother parasite. (**I**) Longitudinal section through one of two daughters at 18 hours plus Shld-1, showing the elongated and lobed appearance of the nucleus. N, nucleus; DG, dense granule; R, rhoptry; C, conoid; MN, micronemes; RB, residual body; Mi, mitochondrion; PP, posterior pole. Scale bars: (A) to (C), 5 μm; (D) to (G), 1 μm; (H) and (I), 0.5 μm.

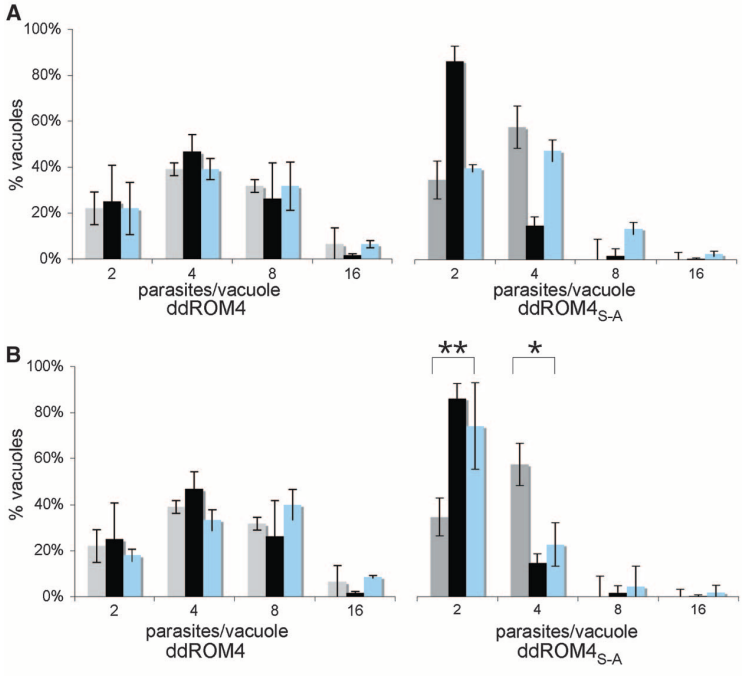

Fig. 3. The dominant negative effect of ddROM4$_{S\text{-}A}$ is reversible and independent of invasion. Intracellular *ddROM4* and *ddROM4$_{S\text{-}A}$* parasites were treated 12 hours with Shld-1 before host cell egress and Shld-1 was then removed 6 hours postinvasion [(**A**), blue bars], or parasites were allowed to invade and Shld-1 was added 6 hours postinvasion and was maintained for the duration of the assay in a total of 18 hours [(**B**), blue bars]. Replication was compared to either nontreated parasites (gray bars) or parasites pretreated 12 hours with Shld-1 before host cell egress and for the time of the assay in a total of 36 hours (black bars). We counted the number of parasites per vacuole 24 hours after invasion. Asterisks indicate statistically significant results (*P = 0.01; **P = 0.004), as determined with the Student's t test. Data are represented as mean ± SD (error bars) of four independent experiments.

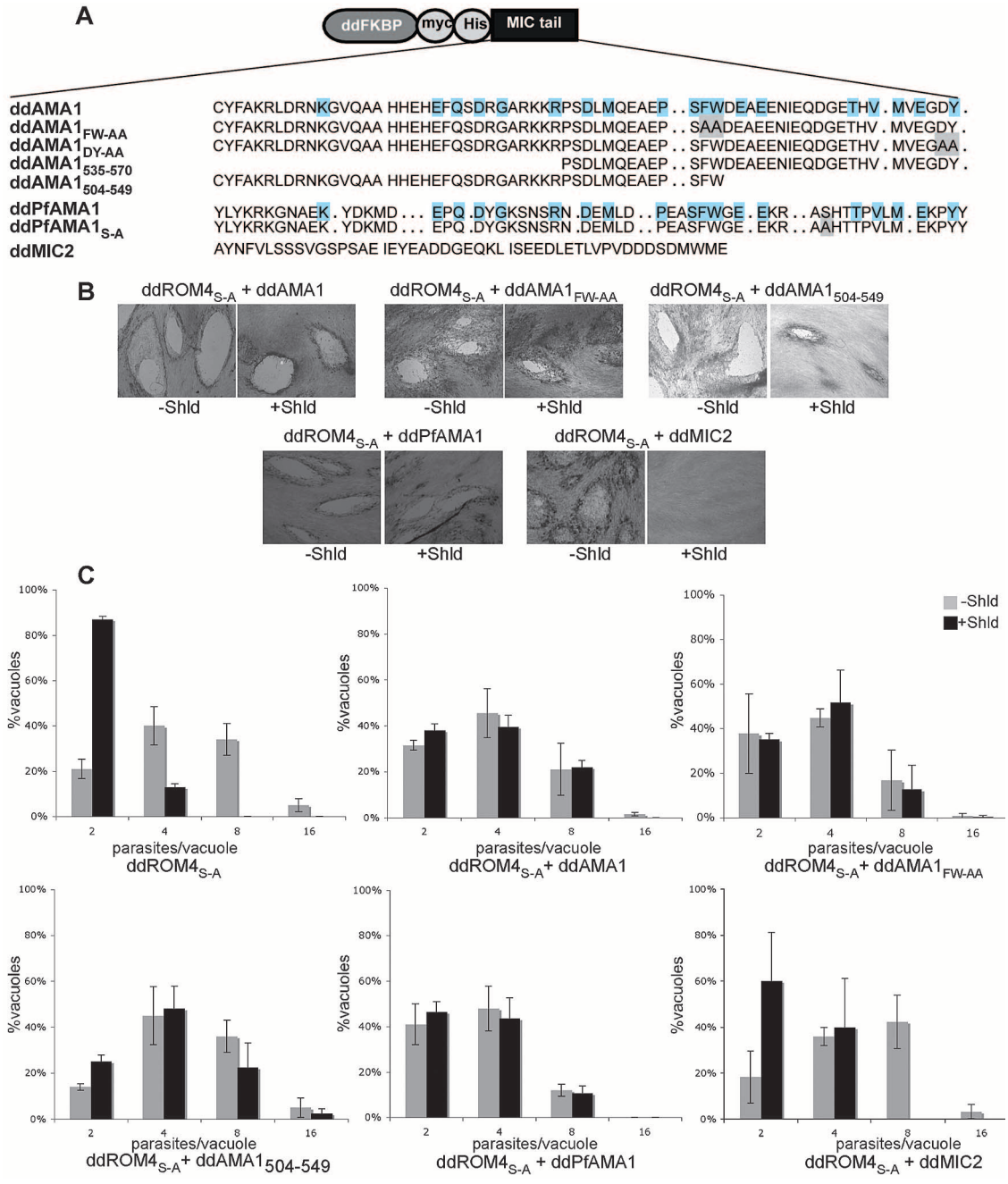

Fig. 4. Expression of ddAMA1, ddAMA1$_{FW-AA}$, and ddPfAMA1 trans-complements the negative effect of ddROM4$_{S-A}$ on replication. (**A**) Schematic of the *T. gondii* (ddAMA1, ddAMA1$_{FW-AA}$, ddAMA1$_{DY-AA}$, ddAMA1$_{535-570}$, ddAMA1$_{504-549}$, and ddMIC2) and *P. falciparum* (ddPfAMA1 and ddPfAMA1$_{S-A}$) fusion proteins used in this study. The sequence of the cloned tails is shown. Mutated residues are boxed in gray; residues conserved in the *T. gondii* and *P. falciparum* AMA1 tail are boxed in blue. (**B**) Plaque assays of *ddROM4$_{S-A}$* parasites expressing ddAMA1, ddAMA1$_{FW-AA}$, ddAMA1$_{504-549}$, ddPfAMA1, or ddMIC2. Parasites were incubated 7 days ± Shld-1 before fixation and Giemsa staining. The assays were only performed simultaneously for the same parasite strain ± Shld-1. (**C**) Intracellular replication assays of parasites expressing ddROM4$_{S-A}$ alone or in combination with ddAMA1, ddAMA1$_{FW-AA}$, ddMIC2, ddAMA1$_{504-549}$, or ddPfAMA1. Parasites were treated 24 hours ± Shld-1 before fixation. Data are represented as mean ± SD (error bars) of three independent experiments.

firming a role for AMA1 in replication. Failure to observe a replication defect in (*15*) may have been the result of an inherent limitation of the system [see supporting online material (SOM)].

The apicomplexan life cycle consists of consecutive transmissive and replicative phases. Premature differentiation into replicative forms would be potentially lethal, so commitment to cell division needs to be tightly regulated in time and space. We show here that a mechanism of regulated intramembrane proteolysis (*27*) acting on AMA1 is implicated in a signaling pathway leading to replication (see SOM). Our study highlights a role in this process for one of the most conserved apicomplexan proteins and shows that this group of parasites has opted to use invasion molecules to couple invasion with replicative growth.

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Supporting Online Material
www.sciencemag.org/cgi/content/full/science.1199284/DC1
Materials and Methods
SOM Text
Figs. S1 to S8
Tables S1 to S3
References

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Big and Mighty: Preverbal Infants Mentally Represent Social Dominance

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Human infants face the formidable challenge of learning the structure of their social environment. Previous research indicates that infants have early-developing representations of intentional agents, and of cooperative social interactions, that help meet that challenge. Here we report five studies with 144 infant participants showing that 10- to 13-month-old, but not 8-month-old, infants recognize when two novel agents have conflicting goals, and that they use the agents' relative size to predict the outcome of the very first dominance contests between them. These results suggest that preverbal infants mentally represent social dominance and use a cue that covaries with it phylogenetically, and marks it metaphorically across human cultures and languages, to predict which of two agents is likely to prevail in a conflict of goals.

Because adequate inferences about social bonds and position confer adaptive advantages (*1*, *2*), infants may posses early-developing mechanisms for representing elementary kinds of social relations, as well as perceptual input analyzers for identifying relevant instances in the social world (*3*–*6*). And indeed, young infants understand intentional action as goal-directed and rational (*7*–*12*). Further, infants represent social interactions among novel agents in terms of whether one helps or hinders the goals of the other, assigning positive valence to a helper and negative valence to a hinderer (*13*, *14*). Slightly older toddlers are intrinsically motivated to help others achieve their goals (*15*), especially when primed with affiliation (*16*). Reflecting the evolved origins of these competencies (*17*), nonhuman primates also understand intentional action (*18*, *19*), recruit the best collaborators (*20*), and sometimes help even human experimenters (*15*).

Affiliative and altruistic interactions reflect one important aspect of human social life. However, group living also entails conflicting goals (*21*) and competition for scarce resources (*22*). Dominance hierarchies that afford dominant animals privileged influence and access to food and mates are ubiquitous among animals, including humans. In fact, across cultures, social hierarchies are found within and between groups (*4*–*6*, *23*). Even toddlers form dominance hierarchies that mirror those found among other primates (*24*, *25*). Moreover, some cues for dominance appear to be (nearly) universal. In particular, dominance rank is associated with relative body size across the animal kingdom, and contestants in dominance fights typically assume postures that maximize their apparent body size until one party subordinates (*6*, *26*–*29*). Increased access to resources as a result of dominance rank may itself lead to increased body size, and some species even show these morphological changes in response to experimental rank manipulations (*27*). Similarly, cultural practices and conceptual metaphors map size and relative height to social hierarchy (*4*–*6*, *29*, *30*). We may speak about a leader as the "big" man, place him on a throne above his subordinates, and kneel before our gods.

If hierarchy is a recurring feature of social environments, early representations of dominance may facilitate the learning process that makes a child a competent cultural member. Here we explore whether preverbal infants form representations of social dominance that mirror any of the evolutionary, cultural, and linguistically widespread features of this concept. Specifically, we established whether infants' attention would be drawn more to unexpected events in which two agents block each other's path of motion and the bigger agent yields to the smaller one by bowing down and moving away, rather than vice versa. To ensure that we tapped general or abstract representations of dominance relations, the agents were novel and the goals minimally defined.

In the first Conflicting Goals experiment, 16 infants between 11 and 16 months of age were familiarized with a series of animations of two blocks of different sizes, each with an eye and a mouth (*31*) (fig. S1). During these familiarization trials, each agent was alone on the platform, bouncing gently from one side to the other, one from right to left and the other in the opposite direction (movies S1 and S2). This was to establish that each agent had the goal of moving to the opposite side of the platform from where it started. An intertrial consisted of both agents simultaneously starting from their habitual beginning positions so that they met in the middle, each blocking the other's habitual path. Then, the agents bumped into each other, backed up, and approached again for a total of three times before they each withdrew (movie S3). This served to highlight the conflict between the two agents' goals being simultaneously realized and to acquaint infants with differences between the familiarization and test trials, such as the simultaneous presence of two agents on the stage and their new patterns of motion, that preceded the crucial experimental manipulations of the test events.

Two test events followed, beginning like the intertrial and with presentation order counterbalanced across participants. In the Expected Outcome test trial, the small agent bowed forward until it was lying down, and then scooted sideways out of the way (away from the viewer), upon which the large agent continued on its path to the end of the stage before the animation froze for 60 s (movie S5). In the otherwise identical Unexpected Outcome test trial, the large agent prostrated itself and yielded the way so that the small agent could complete its path to the end of the stage (movie S4). The time until the infant looked away from the test trials for more than 2 s was measured. In order to be included in the sample, infants must watch the screen during the bowing motion as each agent prostrated itself.

To compare looking times across animations of slightly different lengths, in all experiments we analyzed and report here continued looking times once the animations had frozen to stills (*31*). A repeated-measures analysis of variance (ANOVA) examined the effects of outcome (expected versus unexpected) and presentation order (Big versus Small agent bows first) on looking

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