The moving junction protein RON4, although not critical, facilitates host cell invasion and stabilizes MJ members

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SUMMARY

Toxoplasma gondii is an obligate intracellular parasite of phylum Apicomplexa. To facilitate high-efficiency invasion of host cells, *T. gondii* secretes various proteins related to the moving junction (MJ) complex from rhoptries and micronemes into the interface between the parasite and host. AMA1/RON2/4/5/8 is an important MJ complex, but its mechanism of assembly remains unclear. In this study, we used the CRISPR-Cas9 system to generate a derivative of *T. gondii* strain RH with a null mutation in TgRON4, thought to be an essential MJ component. Deficiency of TgRON4 moderately decreased invasion ability relative to that of the wild-type parasite. In addition, expression of the endogenous *N*-terminal fragment of RON5 decreased in the mutant. Together, the results improve our understanding of the assembly mechanism of the MJ complex of *T. gondii* and raise the possibility of developing new therapeutic drugs that target this complex.

Key words: TgRON4, *Toxoplasma gondii*, knockout, MJ complex.

INTRODUCTION

Toxoplasmosis is a severe zoonotic disease caused by the obligate intracellular parasite *Toxoplasma gondii*. Because *T. gondii* is an opportunistic pathogen, patients with chronic infection usually present with mild clinical symptoms. By contrast, when the infection occurs during pregnancy, the parasite can be vertically transmitted to the fetus through the placenta, potentially leading to abortion, premature birth or stillbirth. Likewise, in immunodeficient patients, toxoplasmosis can cause serious or fatal complications. Moreover, *T. gondii* can invade the brain tissue of immunocompetent hosts, potentially causing changes in some human behaviours (Arling *et al*. 2009; Ling *et al*. 2011; Okusaga *et al*. 2011).

Toxoplasma gondii belongs to phylum Apicomplexa, which also includes *Plasmodium*, the causative agent of malaria. Apicomplexan parasites invade host cells by a conserved, highly efficient mechanism, and invasion can be completed within a few minutes after parasites encounter a suitable host cell. Formation of the moving junction (MJ), a ring-like structure that acts as a bridge to link parasites with target cells, is critical for the invasion by tachyzoites. The MJ is predominantly assembled from proteins secreted from micronemes and rhoptries. Initially, apical membrane antigens (AMAs) are initially secreted from micronemes to the parasite surface prior to invasion, mediating adhesion to the host cell. Subsequently, rhoptry neck proteins (RONs) are secreted and passed through the parasite membrane into the host cells. The ligand–receptor interactions between AMAs and RONs ensure successful host cell invasion, and are required for MJ formation (Boothroyd and Dubremetz, 2008; Lamarque *et al*. 2011).

RON2 is a transmembrane protein that spans the host cell membrane. The *N*-terminus of RON2 resides inside host cells, where it is responsible for binding the remaining members of the MJ complex, RON4/5/8 (Alexander *et al*. 2005; Lebrun *et al*. 2005; Besteiro *et al*. 2009, 2011). Deficiency in TgRON2 leads to severe defects in invasion and replication (Lamarque *et al*. 2011; Tyler and Boothroyd, 2011; Srinivasan *et al*. 2013). Recent functional studies of TgRON8 involved the first complete deletion of a gene encoding MJ protein from *T. gondii*. In TgRON8-deficient parasites, the MJ is frequently unstable, resulting in frequent abortive attachment and invasion of parasites (Straub *et al*. 2011). It was not possible to obtain a knockout strain of TgRON5, which encodes another major component of the MJ complex, suggesting that this gene is essential for life in *T. gondii*. Conditional knockdown of TgRON5 resulted in disordered secretion

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of MJ proteins and severely diminished invasion activity (Beck *et al*. 2014).

RON4 is conserved among apicomplexan parasites. When expression of PbRON4 is blocked in *Plasmodium berghei* via stage-specific deletion of the 3′ untranslated region, cell invasion efficiency of both sporozoites and merozoites is dramatically reduced (Giovannini *et al*. 2011). To date, however, studies of the *T. gondii* homologue of PbRON4 have been limited to characterization of the protein's subcellular location (Lebrun *et al*. 2005; Rashid *et al*. 2011). For many years, TgRON4 was thought to be essential because, as with TgRON5, repeated attempts to knockout this gene were unsuccessful (Alexander *et al*. 2005). Consequently, the roles of TgRON4 in the MJ complex and the mechanism by which TgRON4 facilitates invasion of host cells remain to be determined.

In this study, we used the CRISPR/Cas9 system to engineer a TgRON4-null *T. gondii* strain and evaluated the function of this protein in the MJ complex and host cell invasion. Our results improve our understanding of the formation of the MJ complex and create opportunities to develop new therapeutic drugs.

MATERIAL AND METHODS

Host cell and parasite culture

Vero cells and human foreskin fibroblasts (HFFs) were cultured in complete Dulbecco's modified Eagle's medium (DMEM, Gibco) containing 10% fetal bovine serum (Gibco), 2 mM L-glutamine, 100 U mL^{-1} penicillin and 100 mg mL^{-1} streptomycin (Gibco).

T. gondii (strain RH) tachyzoites lacking a functional hypoxanthine-xanthine-guanine-phosphoribosyl transferase (*HXGPRT*) gene (RH Δ*hxgprt*) were maintained in confluent Vero cells in complete DMEM at 37 °C and 5% CO_2.

Production of recombinant proteins and serum

To generate mouse polyclonal antibodies against TgRON4 and TgRON5, polymerase chain reaction (PCR) products corresponding to residues 26–72 of TgRON4 (TGME49_229010) and residues 249–637 of TgRON5 were amplified from *T. gondii* tachyzoite cDNA and the amplicons were cloned into pGEX-4T-3. The primers used for cloning are listed in Supplementary Table S1. Recombinant proteins were expressed in *Escherichia coli* BL-21DE3 cells and purified. Ten micrograms of purified protein was subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and stained with Coomassie Brilliant Blue. The purified proteins were then mixed with Freund's adjuvant and injected into mice (ICR, SPF) to produce anti-sera. The specificity of the anti-sera was analysed by Western blotting. Anti-SAG2, anti-tubulin and anti-TGGT1_251680 were used as loading controls (the antibodies were generated by immunizing rabbits with recombinant proteins expressed in *E. coli*).

Generation of TgRON4-knockout parasites

To generate a toxoplasma strain with a null mutation in TgRON4 (TGME49_229010), a *TgRON4*-targeting sequence was designed using E-CRISP (E-Crisp.org). A Cas9-control plasmid, named pCD-RON4 (relevant information regarding the vector backbone has been published previously; Zheng *et al*. 2016), was then generated. Briefly, the pCD-RON4 plasmid contains three expression cassettes: Cas9-GFP, TgRON4 guide RNA (gRNA) and dihydrofolate reductase (a map of the vector is shown in Supplementary Fig. S1). RH Δ*hxgprt* parasites (10^7 cells) were transfected with the CRISPR plasmids by electroporation. Cells were selected with 1 μM pyrimethamine 6 h later. The TgRON4-deficient strains were maintained under the control of pyrimethamine for several generations. Next, stable clones (ΔTgRON4-2D10 and ΔTgRON4-2G8) were isolated by limiting dilution in 96-well plates and then screened by Western-blot and PCR analyses. The primers used to characterize the TgRON4-deficient parasites are listed in Fig. 2 and in Supplementary Table S1.

Complementation of TgRON4 in ΔTgRON4 parasites

To express a complementary copy of RON4 in ΔTgRON4 parasites, the coding sequence of TgRON4 was amplified from *T. gondii* genomic DNA using overlap PCR. The TgRON4 gene containing synonymous codons mutated at both target sites for sgRNA (Fig. 7) and fused with a hemagglutinin peptide (HA) tag at the *C*-terminus was ligated into the *Eco*RV site of the pBH vector to generate plasmid pBH-synoRON4-HA, which contains the *HXGPRT* expression cassette. The plasmid was then transfected into ΔTgRON4 parasites and selected with mycophenolic acid (25 μg mL^{-1}) and xanthine (50 μg mL^{-1}). After culture for three generations under the control of these drugs, expression of TgRON4-HA was detected by Western blotting.

Invasion assays

Briefly, freshly egressed ΔTgRON4-2D10 and ΔTgRON4-2G8 parasites were purified by passage through a 27-gauge needle (several times), followed by passage through a 5 μm filter (Sartorius). The parasites were then resuspended in pre-warmed medium, inoculated onto Vero monolayers in 12-

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well plates (10^6 parasites per well), and allowed to invade for 2 h under normal growth conditions (37 °C, 5% CO_2). A strain carrying Cas9-GFP and *HXGPRT*-targeting gRNA was used as a control (Zheng *et al*. 2016). After the monolayers were washed three times with phosphate-buffered saline (PBS) to remove extracellular parasites, the cells were trypsinized for 3 min and collected by centrifugation, followed by another PBS wash. Finally, cells with parasites were resuspended in FACS buffer (1% fetal bovine serum (FBS) in PBS, supplemented with 1 mM EDTA). A total of 10 000 events were counted. Invasion experiments were conducted in triplicate and repeated three times.

The results of invasion assays were confirmed by immunofluorescence analysis (IFA). Monolayers were infected with knockout parasites and controls, fixed with electron microscopy (EM)-grade 3·7% formaldehyde (Biosciences, Inc.), blocked for 1 h at room temperature with 5% bovine serum albumin (BSA) in PBS, and then incubated with rabbit anti-SAG2 antibody for 1 h. After six washes with tris-buffered saline tween (TBST), monolayers were incubated with Alexa Fluor 594-conjugated anti-rabbit IgG, washed six more times with TBST, permeabilized for 30 min with 0·3% Triton X-100/PBS, and then incubated with rabbit anti-SAG2 again. After another six washes, Alexa Fluor 488-conjugated anti-rabbit IgG was added, and the samples were incubated for 1 h. Samples were examined by fluorescence microscopy. Cells stained both green and red were scored as attached but uninvaded parasites, whereas those stained only in green were scored as internalized parasites. Ten microscopic fields were randomly counted for each well. Experiments were conducted in triplicate and repeated at least thrice.

Egress assays

For egress assays, parasites were collected, purified as described above, and counted. The parasites were then injected into Vero monolayers (10^5 parasites per well), and cultured under normal growth conditions for 32 h. Extracellular parasites were washed three times with PBS, and then incubated with 3 μM A23187 (Sigma), a calcium ionophore, diluted in Hank's Balanced Salts Solution. After 5 min incubation at 37 °C, parasites were fixed and IFA was performed with rabbit anti-SAG1 and mouse anti-GRA7. A total of 100 vacuoles per field were counted, and egress events were scored under a microscope.

Replication assays

Vero monolayers in 12-well plates were infected with parasites (10^6 parasites per well). Invasion was allowed to proceed for 2 h under normal growth conditions (37 °C, 5% CO_2), extracellular parasites were washed away with PBS, and the parasites were incubated for another 24 h. To investigate the replication ability of knockout parasites, the parasites were counted in 100 vacuoles. Experiments were conducted in triplicate and repeated three times.

Statistical analysis

Data from the attachment, invasion, replication and egress assays were analysed using an unpaired Student's *t*-test. A *P*-value <0·05 was statistically significant.

RESULTS

Generation of recombinant proteins and polyclonal antibodies

To generate antibodies against TgRON4 and TgRON5, partial sequences of TgRON4 and TgRON5 fused with glutathione S-transferase (GST) tags were expressed in *E. coli*. The recombinant proteins were purified successfully as shown in Fig. 1A. Recombinant TgRON5 resolved as multiple bands, which might represent degradation products. The anti-sera produced in mice recognized native TgRON4 and TgRON5 proteins on Western blotting (Fig. 1B) and IFA (Fig. 3B and Supplementary Fig. S2). The bands in the Vero cell lysate might be derived from non-specific reactions.

Construction of TgRON4-knockout parasites using the CRISPR-Cas9 system

We first sought to determine the importance of TgRON4 in MJ in parasite invasion. To this end, we generated *TgRON4*-knockout parasites in the RH Δ*hxgprt* strain. The *TgRON4*-targeting sequence is shown in Fig. 2A. The sgRNA targeted two sites within the TgRON4 locus because they contain repeat sequences. After selection with pyrimethamine for several passages, parasite clones stably expressing Cas9-GFP were obtained, and two clones were selected (designated ΔTgRON4-2D10 and ΔTgRON4-2G8) for further experiments. Next, we performed PCR to amplify the targeted RON4 locus; however, we failed to obtain PCR products when using several pairs of primers specific for the RON4 locus (Fig. 2B). This may be because a large foreign fragment (most probably derived from the transfected plasmids) became integrated into the locus. Therefore, another set of primers specific for Cas9 and dihydrofolate reductase (DHFR) sequences within the plasmids were designed and used to amplify the products (Fig. 2C) and for sequencing. We found that both sgRNA-targeting sites in the RON4 locus were edited. An approximately 5700 bp fragment was inserted into the first targeting site (1106–1124 bp), and five base pairs were deleted from the second targeting site (2006–2024 bp) (Fig. 2D).

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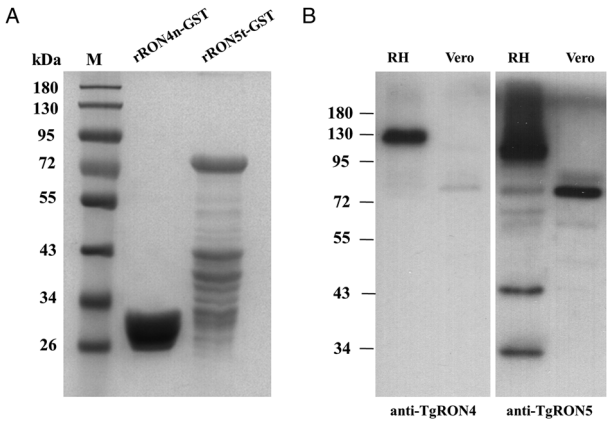

Fig. 1. Generation of recombinant proteins and anti-serum. (A) SDS-PAGE analysis of purified recombinant TgRON4 and TgRON5 proteins (10 μg of each protein was loaded on the gel and stained with Coomassie Brilliant Blue). (B) Western-blot analysis of the specificity and immunogenicity of the corresponding anti-sera. Lysate of Vero cells served as a negative control.

Depletion of TgRON4 from the knockout mutants was also confirmed by Western blotting (Fig. 3A) and IFA (Fig. 3B).

Deletion of TgRON4 affects expression of TgRON5N

We next asked how TgRON4 contributes to the stability of MJ members. An antibody against TgRON5N was used to detect expression of native TgRON5. Only one band, corresponding to TgRON5N, was observed on the blots. Interestingly, the expression level of TgRON5N was clearly reduced in TgRON4-deficient parasites (Fig. 4A and B).

Loss of TgRON4 affects invasion of host cells

We then determined the invasion ability of TgRON4-null parasites by flow cytometry. In these experiments, Vero monolayers were infected and incubated for 2 h. The invasion rates of ΔTgRON4-2D10 and ΔTgRON4-2G8 were significantly lower than that of wild-type parasites (Fig. 5A). Thus, TgRON4 is required for efficient invasion of host cells by *T. gondii*. To confirm the results, we observed the internalized parasites by fluorescence microscopy. Again, both TgRON4-deficient strains exhibited a significant decrease in the invasion rate (Fig. 5B). These results indicated that, although loss of TgRON4 is not fatal, the mutation reduces the ability of *T. gondii* to invade host cells *in vitro*, rather than by impairing attachment.

Loss of TgRON4 does not affect growth of parasites in host cells or egress efficiency

To determine whether *TgRON4*-knockout parasites have defects in intracellular growth, we performed growth assays in which we allowed the parasites to internalize for 24 h and then scored the number of parasites per vacuole. The replication abilities of both knockout parasites were similar to that of the Cas9 control parasites (Fig. 6A). These results demonstrate that TgRON4 is dispensable during the division of parasites in host cells. Furthermore, upon triggering with calcium ionophore, the egress ability of knockout parasites was unaffected (Fig. 6B).

Complementary expression of synoTgRON4 by ΔTgRON4-2G8 parasites restores invasiveness

To further confirm the effect of TgRON4 in invasiveness, we generated a complementary TgRON4 expression plasmid by replacing the original codons with synonymous codons, designated pBH-synoTgRON4-HA (Supplementary Fig. S5). Western-blot analysis with anti-RON4 and anti-HA antibodies revealed proper expression of TgRON4-HA (Fig. 7A). The invasiveness of *ΔTgRON4-2G8* was restored by complementary expression of TgRON4 (Fig. 7B). Furthermore, we observed that complementary expression of TgRON4 restored the expression level of TgRON5N (Fig. 7C and D).

DISCUSSION

Efficient invasion by apicomplexan parasites is facilitated by the formation of the MJ complex. Several secretory factors, including microneme and rhoptry proteins, assemble to form these special structures. Although the anchor function of the core members of the MJ complex has been extensively studied, additional research on the main members of the complex will improve our understanding of the mechanism of host cell invasion.

First, we generated polyclonal antibodies specific for TgRON4 and TgRON5. Although the Vero cell lysate showed non-specific reactions, the sizes of the non-specific bands were different from those of the target bands on immunoblots. We also conducted indirect fluorescent antibody test (IFAT) using the same antibodies. The antibodies recognized the native proteins specifically in parasites. We think, therefore, that the appearance on non-specific bands does not alter our interpretation of the data in this study. To investigate the role of TgRON4 in the MJ complex, we used the CRISPR/Cas9 system to generate a *TgRON4*-deficient mutant in *T. gondii* type I strain, RH, which is proven to be an extremely efficient system for targeted gene disruption (Shen *et al.* 2014; Zheng *et al.* 2015). A previous study failed to delete the *RON4* gene by replacement with the *HXGPRT* selectable marker (Alexander *et al.* 2005). However, we obtained the *TgRON4*-

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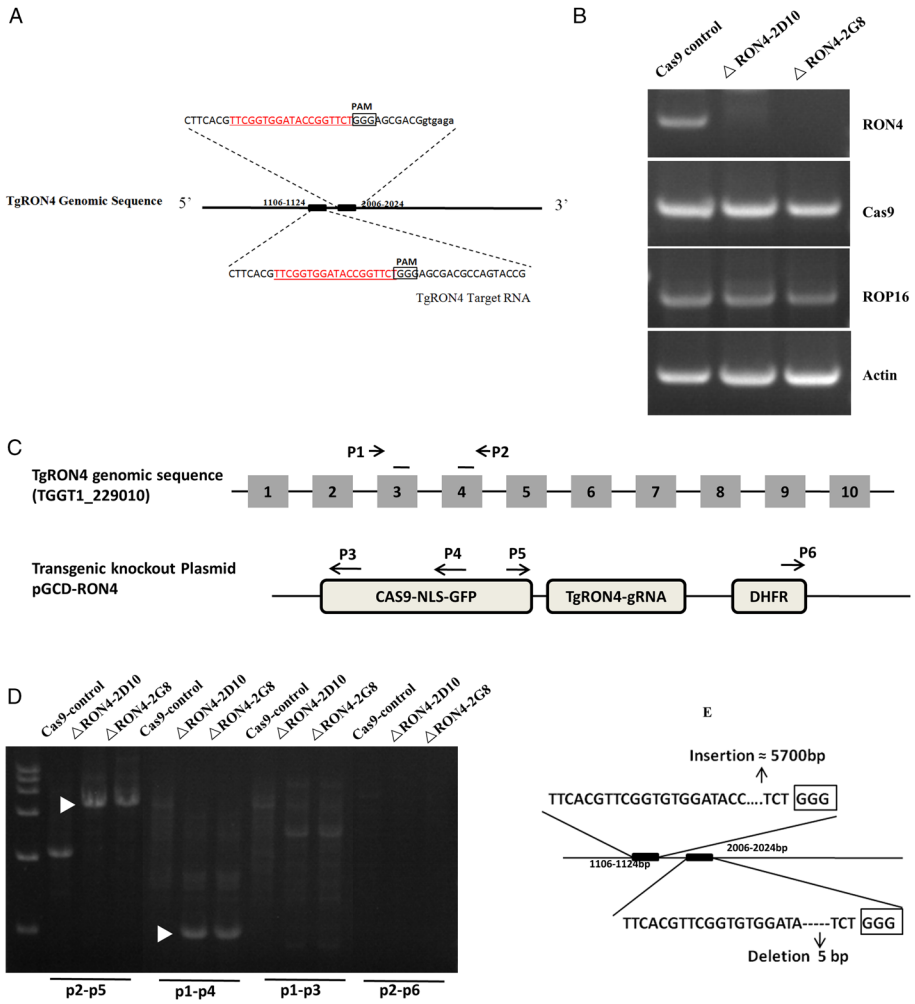

Fig. 2. Schematic illustration of TgRON4 knockout of plasmids. (A) Targeting sequence for TgRON4. The sequence encoding the gRNA targeting TgRON4 is underlined. (B) PCR analysis of ΔTgRON4 parasites. The primers were designed to span the target sequence of the gene knockout. ROP16, GRA16, actin and Cas9 served as loading controls. (C) The position of the primers used to identify genome editing within the RON4 locus. (D) PCR confirmed mutations in the RON4 locus. (E) Gene editing within the RON4 locus of ΔTgRON4 parasites.

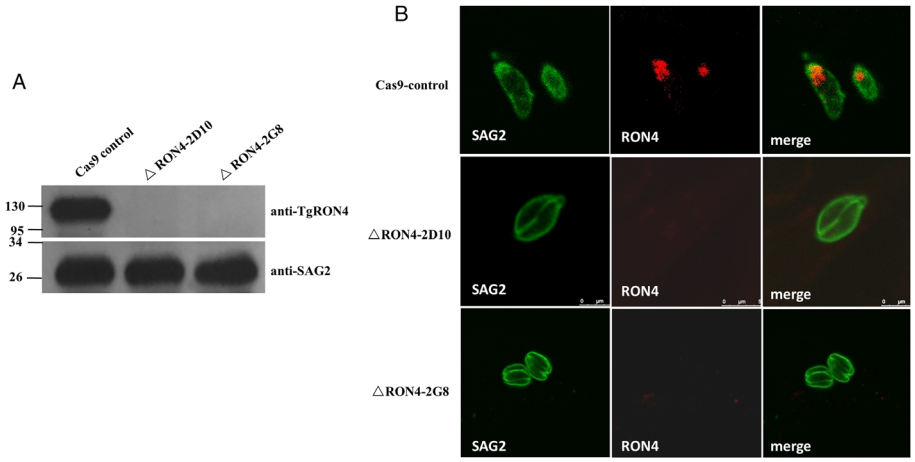

Fig. 3. Western-blot and immunofluorescence analysis of ΔTgRON4 parasites. (A) Western-blot analysis of lysates derived from Cas9 control parasites and ΔTgRON4 parasites. Blots were probed with anti-TgRON4 serum raised in mice. SAG2 served as a loading control. (B) Immunofluorescence analysis of Cas9 control parasites and ΔTgRON4 parasites reveals the absence of RON4 staining at the rhoptry necks of knockout parasites.

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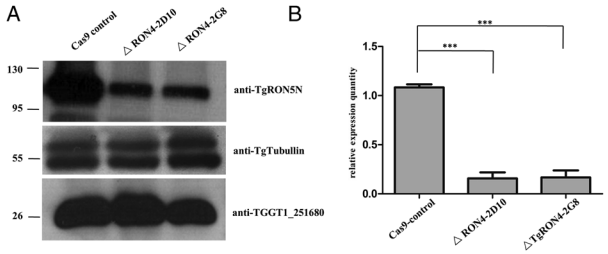

Fig. 4. Deletion of TgRON4 affected the expression level of TgRON5N. (A) Western-blot analysis of TgRON5 expression in TgRON4-deficient parasites. Blots were probed with anti-RON5N serum raised in mice. Tubulin and TGGT1_251680 served as loading controls. (B) Relative expression measured by Western-blot analysis (A).

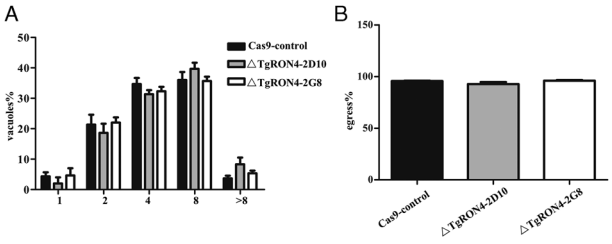

Fig. 6. TgRON4 is dispensable for replication in and egress from host cells. (A) Replication of ΔTgRON4 parasites was examined by microscopy. At least 100 vacuoles were scored for each condition. (B) The egress ability of ΔTgRON4 parasites was evaluated under stimulation with calcium ionophore III. At least 100 vacuoles were scored for each condition.

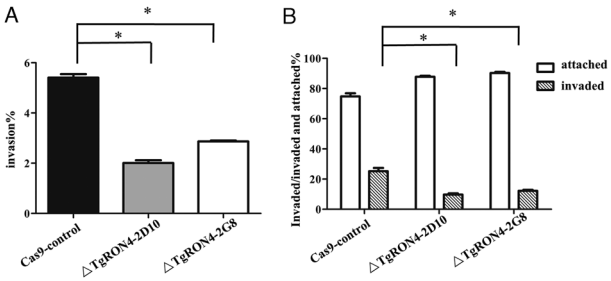

Fig. 5. Loss of TgRON4 affects invasion into host cells but is dispensable for growth of parasites within parasitophorous vacuole (PV). (A) Flow-cytometric analysis of the invasion ability of ΔTgRON4 parasites. (B) Observation of invasion ability of ΔTgRON4 parasites under the microscope. At least 10 fields (representing more than 200 parasites) were counted per group.

deficient strain with ease, possibly owing to the high efficiency of the knockout method we employed. However, we cannot exclude the possibility that RON4 might play different roles in different parasites, or that other proteins might compensate for the function of TgRON4 in *T. gondii*. RON4 of *P. berghei* is critical for sporozoite invasion of hepatocytes and merozoite invasion of erythrocytes (Giovannini *et al*. 2011), and it remains to be determined whether TgRON4 is essential for other stages of *T. gondii* invasion.

A previous study (Beck *et al*. 2014) showed that the stability and localization of TgRON2 are impaired when expression of TgRON5 is disrupted, suggesting that TgRON5 is required to maintain the stability of the whole complex. We observed that expression of TgRON5N decreased in TgRON4-deficient parasites. The data might reflect the possible instability of TgRON5N due to a defect in targeting the RON complex to the rhoptry neck or MJ in the absence of RON4, as was observed for the RON5-deficient mutants. However, evidence is lacking. We observed TgRON5 in TgRON4-deficient parasites using immunofluorescence assays (Supplementary Fig. S2). TgRON5 (at least TgRON5N) was targeted to the neck of rhoptry; however, precise localization should be confirmed by co-staining using a RON protein marker such as RON11 and an anti-TgRON5 antibody. At the same time, targeting TgRON5 to the MJ remains to be confirmed. Future studies should monitor expression and localization of other MJ members, including TgAMA1, TgRON2, TgRON5C and TgRON8, in TgRON4-deficient parasites to improve our understanding of the function of TgRON4 in the MJ. However, TgRON5 is subjected to proteolytic processing to yield RON5N and RON5C, although this is not required for parasite invasion (Beck *et al*. 2014). Although it is not clear, we observed slight accumulation of unprocessed TgRON5 in overexposed blots. Therefore, another possible explanation for the reduction of TgRON5N is that the proteolytic processes in TgRON4-deficient parasites were dysfunctional.

These data indicate that loss TgRON4 affects the ability to invade host cells, and that compensating for TgRON4 restores this ability. These data suggest a role for TgRON4 in the invasion process. However, the impaired invasion ability of TgRON4-deficient parasites might also be due to the instability of other MJ members, or to incomplete targeting of the whole RON complex at the neck of rhoptry or in the MJ. Alternatively, digestion of TgRON5 might be necessary for invasion of parasites when TgRON4 is absent. In addition, a previous study characterized the interaction between TgRON4 and the host cell cytoskeleton in parasite-infected cells (Takemae *et al*. 2013), and we cannot exclude the possibility that the impaired invasion ability of TgRON4-deficient parasites is due to diminished interaction between TgRON4 and the cytoskeleton, or some other phenomenon.

RON2 has two functional paralogs (RON_{2L1} and RON_{2L2}), which form functional invasion complexes along with paralogs of AMA1 (AMA4 and AMA3, respectively). Under normal conditions, these two complexes play important roles only during

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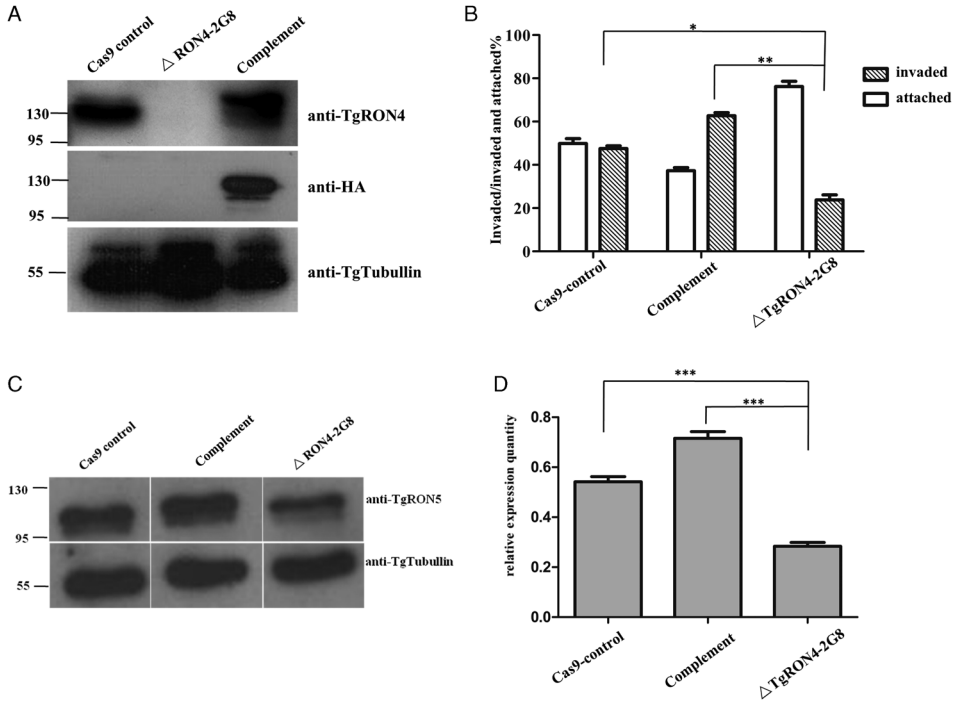

Fig. 7. Compensating for TgRON4 restores the invasive ability of parasites and stability of RON5. (A) Western-blot analysis of TgRON4 protein on parasites harbouring synonymous codons (synoTgRON4). Tubulin served as a loading control. (B) Examination of the invasive ability of ΔTgRON4-2G8/synoTgRON4 parasites. (C) Western-blot analysis of TgRON5 expression in TgRON4-deficient parasites and complementary parasites. (D) Relative expression measured by Western-blot analysis.

sporozoite invasion, analogous to RON2/AMA1 in tachyzoites (Poukchanski *et al*. 2013; Lamarque *et al*. 2014; Parker *et al*. 2016). Determining whether TgRON4 interacts with $TgRON_{2L1}$ or $TgRON_{2L2}$ will provide further insight into the mechanism of *T. gondii* invasion.

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SUPPLEMENTARY MATERIAL

The supplementary material for this article can be found at https://doi.org/10.1017/S0031182017000968.

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