RON4L1 is a new member of the moving junction complex in *Toxoplasma gondii*

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Apicomplexa parasites, including *Toxoplasma* and *Plasmodium* species, possess a unique invasion mechanism that involves a tight apposition between the parasite and the host plasma membranes, called “moving junction” (MJ). The MJ is formed by the assembly of the microneme protein AMA1, exposed at the surface of the parasite, and the parasite rhoptry neck (RON) protein RON2, exposed at the surface of the host cell. In the host cell, RON2 is associated with three additional parasite RON proteins, RON4, RON5 and RON8. Here we describe RON4L1, an additional member of the MJ complex in *Toxoplasma*. RON4L1 displays some sequence similarity with RON4 and is cleaved at the C-terminal end before reaching the rhoptry neck. Upon secretion during invasion, RON4L1 is associated with MJ and targeted to the cytosolic face of the host membrane. We generated a RON4L1 knock-out cell line and showed that it is not essential for the lytic cycle in *vitro*, although mutant parasites kill mice less efficiently. Similarly to RON8, RON4L1 is a coccidian-specific protein and its traffic to the MJ is not affected in absence of RON2, RON4 and RON5, suggesting the co-existence of independent MJ complexes in tachyzoite of *Toxoplasma*.

Apicomplexa parasites are responsible for important human and animal diseases. The phylum includes significant human pathogens such as *Plasmodium* species responsible for malaria, or *Toxoplasma* the agent of toxoplasmosis. The invasion process is a crucial step for these obligatory intracellular parasites and is mostly conserved throughout the phylum. In most cases, it involves the formation of a unique feature called Moving Junction (MJ). The MJ is a tight apposition between the host cell and parasite plasma membranes. This structure has been first observed in 1978 by electron-microscopy of *Plasmodium knowlesi* merozoites entering red blood cells1, then its molecular characterization started being unraveled in *Toxoplasma* tachyzoites (the invasive form of the parasite responsible for the acute phase of the disease) almost 30 years later. The molecular components of the MJ are Apicomplexa-specific proteins2–5 secreted from two distinct apical organelles of the parasite called micronemes and rhoptries, the latter exhibiting a peculiar club-shape structure with a thin duct (or neck) and a bulbous part6. During invasion, the parasite translocates a microneme protein, the apical membrane antigen 1 (AMA1), at its own plasma membrane, and exports a rhoptry neck complex (composed of RON2, RON4, RON5 and RON8 proteins) into the host cell7. It should be noted that RON8 is not universally conserved, but seems specific to coccidian parasites such as *Toxoplasma, Eimeria* and *Neospora*5. RON2 is inserted into the host plasma membrane and exposes a short segment at the surface of the host cell which interacts with AMA18. AMA1 and RON2 thus form an intimate contact9,10 which generates a close and irreversible interaction between the parasite and the host cell11.

RON, RON5 and RON8 are soluble proteins, tethered to RON2 and exposed to the cytosolic face of the host cell membrane where RON2, RON4 and RON5 cooperatively recruit host adaptor proteins that might contribute to anchor the parasite to the host cytoskeleton11. Parasites lacking RON411 or RON812 are viable but severely impaired in invasion. In contrast, it has not been possible to generate knock-out mutants for RON212 and RON513, indicating a more critical role during invasion for those proteins. Conditional knock-down (KD) mutants for RON2 and RON5 exhibit an invasion defect of around 90%14,15, while KO-RON4 and KO RON8 exhibit 60% and 70% reduction of invasion, respectively. In absence of RON8, the remaining MJ components RON2/RON4/RON5 are correctly targeted to the rhoptries and to the MJ, but 20% of parasites leave trails connecting the PVM with the

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host cell plasma membrane after invasion. This suggests that RON8 could be required for an efficient fission of the PVM at the end of invasion. In contrast, RON2, RON4 or RON5 depletion induces expression and targeting defects of other members of the MJ complex, resulting in fact in triple functional mutants, hence limiting the phenotypic resolution of individual RON functions. In KD-RON2, KD-RON5 and KO-RON4 parasites, RON8 correctly localizes to the rhoptries and is found associated with the MJ during invasion in successful invaders. This indicates that an alternate MJ complex might be formed in absence of RON2, RON4 and RON5 in Toxoplasma tachyzoites, also possibly containing additional components. Recent studies showed that a MJ can also be formed in absence of AMA1 in tachyzoites. Deciphering the architecture of the MJ in complete absence of AMA1 has shown that tachyzoites knock-out for AMA1 upregulate homologs of AMA1 and RON2 that cooperate to support residual invasion. Not only this highlighted a likely functional redundancy in MJ components, but it also suggested some variety in MJ proteins, hinting that additional components remained to be discovered. RON4, unlike RON5 and RON8, possesses a putative homolog coded by a gene which was previously named RON4L1 (www.Toxodb.org accession number TTGG1_253370). This gene displays a transcription profile that peaks during the S/M phase, a common characteristic of genes coding for rhoptry proteins. Apart from transcriptional evidence for the RON4L1 gene, nothing was known about the actual localization or role of the corresponding protein in the parasite.

In this report, we further investigate the molecular composition of the MJ complex by characterizing a new rhoptry neck protein, RON4L1, that shares sequence homology with RON4 and is highly expressed in tachyzoites. We show that RON4L1 is a new member of the MJ complex that is present at the MJ and exposed at the cytosolic face of the host membrane during invasion. We successfully generated a direct knock-out of the RON4L1 gene. RON4L1-depleted parasites invade cells similarly as control in vitro but are significantly impaired in virulence in mice, a defect restored by complementation with an additional RON4L1 copy. When RON4, RON2 and RON5 are down-regulated, RON4L1 expression and its localization in the neck of the rhoptry in intracellular parasites, or at the MJ during invasion, are unchanged. A characteristic ring shape labelling of RON8 and RON4L1, observed in remaining invaders, supporting the existence of an alternate, coccidian-specific, complex for invasion independent of the main and more conserved among Apicomplexa RON2/RON4/RON5 complex. Taken together, our results offer a better understanding of the MJ architecture and support the existence of functional and independent MJ complexes in tachyzoites of T. gondii.

Results
RON4L1 is a new rhoptry neck protein. Transcriptomic and proteomic datasets in ToxoDB indicate that RON4L1 is expressed in tachyzoite, bradyzoite and sporozoite stages, as well as in the cat enteropthelial stage (www.Toxodb.org). Like RON8, RON4L1 is a coccidian-specific protein. RON4L1 shows 13% identity and 21% similarity with RON4 (Fig. S1): RON4L1 is noticeably longer than RON4, and essentially homologous to its C-terminal region. RON4L1 is a 1981 amino acids long protein, with a predicted molecular mass of 216 kDa. Besides a predicted signal peptide, no transmembrane domain or any other known recognized domains can be identified.

To localize RON4L1, we first tagged the protein with a triple hemagglutinin tag (HA3) at the C-terminal end, by single recombination at the endogenous locus, as described previously and represented in Fig. 1a. A clone was selected and verified by PCR for proper construct integration (Fig. 1b). IFA using anti-HA antibodies revealed that only a fraction of the parasites display a HA3-tag signal, although when present, the signal observed was reminiscent of the pre-rhoptry compartments of developing parasites (data not shown). Because many rhoptry proteins are processed in the secretory pathway in the transition step between immature (pre-rhoptry) and the mature organelle, this labelling suggested that RON4L1 is a rhoptry protein potentially undergoing a C-terminal proteolytic maturation. To verify this hypothesis, we first performed co-localization with the pre-rhoptry marker Pro-ROP4. When tagged at its C-terminal-end, RON4L1 co-localizes perfectly with the immature ROP4 protein stored in the pre-rhoptries (Fig. 1c, upper panel), while it is absent of mature organelles detected with anti-RON2 antibodies (Fig. 1c, lower panel). This indicated that RON4L1 likely travels through the secretory pathway, is addressed to the pre-rhoptries and undergoes a C-terminal cleavage during trafficking to mature organelles. To confirm this hypothesis, we then added a tag at the N-terminus part of the protein in order to follow the mature protein. A strategy was designed to insert a HA3-tag after the signal peptide of RON4L1 in the Δku80 RH strain by CRISPR/Cas9-mediated genome editing (Fig. 2a) using a specific donor sequence (Fig. S2) as a template for homologous recombination. Transfected parasites were sorted by FACS to enrich the Cas9-YFP transfected population and then cloned immediately. PCR amplifications (Fig. 2b) and sequencing confirmed the insertion of the HA3 tag after the signal peptide of RON4L1 in a clone referred hereafter as HA3-RON4L1, by opposition to the C-terminal tagged RON4L1-HA3 strain. HA labelling of HA3-RON4L1 parasites delineated the pre-rhoptries in some parasites, as observed for RON4L1-HA3 parasites (Fig. 2c upper panel). However, in contrast to the C-terminal tagging, the remaining HA3-RON4L1 parasites displayed an apical labeling that co-localized perfectly with the rhoptry neck marker RON2 (Fig. 2c lower panel).

Western blot analysis with anti-HA antibody showed that a faint high molecular weight product was observed in protein extracts from the C-terminus tagged cell line, likely corresponding to the unprocessed form (Fig. 2d). While this form was also detected in lysates from the N-terminus tagged cell line, the main product had a lower molecular mass (Fig. 2d). Several lower bands, possibly corresponding to further processing or degradation products, were also detected (Fig. 2d).

In conclusion, RON4L1 is a new rhoptry neck protein that undergoes a C-terminal cleavage during rhoptry maturation (Fig. 2e).

RON4L1 is a new component of the MJ RON complex exposed to the cytosolic face of the host cell during invasion. In order to know if RON4L1 is secreted during invasion, we performed IFAs on
HA3-RON4 L1 parasites using anti-HA antibodies, with permeabilization conditions optimized to detect only the material secreted by the parasite. Indeed, with low saponin concentration, rhoptries membranes are not permeabilized, and antibodies detect the protein only once secreted from the organelle. On invading parasites, the RON4 L1 labelling was circumferential, coincided with the constriction of the parasite, and co-localized with the MJ marker RON2 (Fig. 3a, panels 1 and 2). After complete internalization, RON4 L1 co-localized with RON2 as a puncta systematically observed at the posterior end of the parasite, which corresponds to the residual MJ (Fig. 3a, panel 3). Overall, our results indicate that RON4 L1 is part of the MJ.

To test whether RON4 L1 physically interacts with the RON2/RON4/RON5/RON8 MJ complex, we performed an immunoprecipitation using anti-HA beads on a HA3-RON4 L1 parasites lysate. All components of the MJ complex (RON2, RON4, RON5, RON8 and AMA1) were co-immunoprecipitated together with RON4 L1, even in stringent experimental conditions (1 M NaCl washes) (Fig. 3b), suggesting a strong interaction.

Finally, we sought to test the topology of RON4 L1 at the MJ. We were unable to identify any putative transmembrane domains in RON4 L1 using prediction software such as TMHMM (http://www.cbs.dtu.dk/services/TMHMM) or PredictProtein (https://www.predictprotein.org). We thus suspected the protein might be associated with RON4, RON5 and RON8 on the cytoplasmic side of the host cell. To test this possibility, we used the “glass-bead loading” approach previously described to demonstrate the export of RON4, and their association with the cytosolic face of the host membrane. The design of this experiment, based on cytoplasmic loading of antibodies within the host cell prior to invasion, allows the detection of RON4 L1 at the MJ only if the protein is secreted into the host cell cytoplasm (see Methods). This is illustrated by the left panel of Fig. 3c, in which one parasite is extracellular (SAG1 positive, right), while the second is intracellular (SAG1 negative, left) and the latter is also stained with RON4 L1. This characteristic signal corresponding to the residual MJ shows that RON4 L1 is exposed on the cytoplasmic side of the host cell. In the same experimental conditions, for invading parasites (Fig. 3c, right panel) RON4 L1 was also detected at the ring-like MJ.

Figure 1. Epitope tagging of RON4 L1. (a) Scheme illustrating the approach used to endogenously tag RON4 L1 at the C-terminus. Primers used to verify the genetic modification and the size of the PCR product are indicated. (b) The HA3 tagging at the 3′end of RON4 L1 locus by single homologous recombination was verified with PCR using primers P1 and P2, whose positions are indicated in (a). (c) IFA on intracellular Δku80 RON4 L1-HA3 parasites using anti-pro-ROP4 and anti-HA antibodies (upper panel) or anti-RON2-4 antibodies (lower panel). The anti-HA antibodies stains only the pre-rhoptries. Scale bars, 5 μm.
From these experiments, we conclude that RON4L1 is a new member of the MJ complex that is exposed on the cytoplasmic face of the host cell, together with RON2, RON4, RON5 and RON8 (Fig. 3d). RON4L1 is correctly targeted to the neck of the rhoptry in KD-RON4 and remains associated with the MJ on invading parasites.

In KD-RON2, KD-RON4 and KD-RON5 mutants, the formation of the RON2/RON4/RON5 complex is disrupted 12,13,16, while RON8 is expressed in normal amount and traffics correctly to the MJ. We thus sought to investigate the fate of RON4L1 in a parasite where the formation of the RON2/RON4/RON5 complex is affected. To this end, we introduced a triple HA epitope-tag after the signal sequence of RON4L1 in the KD-RON4 background (Fig. S3), using the same strategy as described in Fig. 2a. As previously observed, altering RON4 expression leads to a significant decrease of RON2 and RON5 expression (Fig. 4a). In contrast, and similarly to RON8, RON4 L1 is expressed at normal levels (Fig. 4a) and correctly associated with rhoptries in intracellular parasites (Fig. 4b) and at the MJ in invading parasites (Fig. 4c). In conclusion, similarly to RON8, RON4 L1 traffic seems independent of RON2, RON5 and RON4 proteins expression and complex formation.

RON4L1 plays a role in virulence in a mouse model. To test the function of RON4L1, we used a CRISPR/Cas9 strategy to generate a knock-out cell line by replacing a 22.2 kbp genomic region encompassing the RON4L1 open reading frame by a DHFR cassette in the HA3-RON4L1 cell line background (Fig. 5a). Knock-out parasites (KO-RON4L1) were obtained, as verified by diagnostic PCR (Fig. 5b), western blot (Fig. 5c) and IFA (Fig. 5d and Supplementary Fig. S4). This demonstrates RON4L1 is not required for parasite survival in vitro. While RON4L1 is not detected at the MJ of KO-RON4L1 parasites, remaining members of the RON complex (RON2, RON4, RON5 and RON8) are still expressed at normal levels (Fig. 5e). The localization of these proteins also remains unaltered: they are present in the neck of the rhoptries in intracellular parasites (Fig. 5f) and at the MJ during invasion (Fig. 5d).

To test the consequence of the loss of RON4L1 on the parasite lytic cycle, we then performed a plaque assay, and found that the size of lysis plaques was similar between wild-type and KO-RON4L1 parasites (Fig. 6a). Besides,
no apparent defect of invasion was detected in fibroblastic cells (Fig. 6b). We next assessed if the loss of RON4 L1 could impact parasite virulence in the mouse model (Fig. 6c). The mortality of mice infected with KO-RON4 L1 was delayed (4 to 5 days) compared with that of mice infected with parasites of Δku80 parental strain or with a cosmid-complemented RON4 L1-reexpressing cell line (Cpt-RON4 L1) (Supplementary Fig. S4). These results show that even if the loss of RON4 L1 is not significantly impacting in vitro growth, the protein contributes to parasite virulence in vivo.

Discussion
The formation of the MJ is an essential mechanism for many Apicomplexan parasites including Plasmodium and Toxoplasma species. In Toxoplasma, the parasite MJ components are composed of AMA1, a micronemal protein exposed on the surface of the parasite, and of a complex of rhoptry neck proteins comprising RON2, RON4,
RON5 and RON8. In the present study, we identify a new rhoptry neck protein, RON4_{L1}, associated with the MJ complex. RON4_{L1} has been identified through its partial homology with the RON4 protein. In contrast to the paralogs of RON2, RON2_{L1} and RON2_{L2} which are preferentially expressed in sporozoites or bradyzoites, and up-regulated upon depletion of AMA1', RON4_{L1} is abundant in tachyzoites and is not up-regulated in the KD-RON4 mutant. Thus, RON4_{L1} is a constitutive component of the MJ complex in tachyzoites.

While RON2, RON4 and RON5 are conserved in most Apicomplexa, RON8 and RON4_{L1} are specific to Coccidia. Little is known about how RON proteins traffic to the rhoptry neck. RON5 is believed to be important for RON2 stability and RON4 targeting. Similarly, RON2 and RON4 also play a role in stabilizing the RON2/RON4/RON5 complex. Thus, depletion of either RON2, RON4 or RON5 is sufficient to reduce the expression and localization of the others. In contrast, their depletion does not influence the behavior of the coccidian-specific MJ members RON8 and RON4_{L1} (this study). Homologs of RON2 and RON4 and RON5 can be found in many Apicomplexa parasites, supporting the idea of a conserved RON2/RON4/RON5 core complex, and the existence of additional members which would be species specific. Both RON8 and RON4_{L1} are

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**Figure 4.** RON4_{L1} expression and localization is independent of the RON2/4/5 complex. (a) Western blot analysis of Δku80 HA_{3}-RON4_{L1} and KD-RON4 HA_{3}-RON4_{L1} parasite lysates treated with ATc for 48 hours using anti-HA, anti-RON2-3, anti-RON4 antibody, anti-RON5 and anti-RON8 antibodies. RON4_{L1} expression is not affected by RON2, RON4 and RON5 depletion. ROP5: loading control. Full-length blots are presented in Supplementary Fig. 6. (b) IFA of intracellular KD-RON4 parasites in which RON4_{L1} has been HA_{3}-tagged. RON4_{L1} localizes in the neck of the rhoptries, as shown by the co-localization with RON2. Scale bar, 2µm. (c) IFA of invading KD-RON4 HA_{3}-RON4_{L1} parasites. RON4_{L1} remains present at the moving junction in invading KD-RON4 parasites. Scale bar, 2µm. DIC: differential interference contrast.
soluble proteins exposed to the cytosolic face of the MJ (Fig. 3d). How they associate with the MJ during the progression of invasion in the absence of the core complex remains unknown. This is particularly intriguing, because so far RON2 is the only known membrane anchor for the RON complex in tachyzoites: it is inserted as a transmembrane protein into the host cell surface, and is supposed to serve as a host connector (via AMA1) to the gliding machinery necessary for the progression of internalization. The localization of both RON8 and RON4 L1 to a MJ-like ring during invasion in absence of RON2 suggests additional RONs might compensate the anchoring function. Whether RON2 L1, which is normally expressed at very low level in tachyzoite, could be such a candidate, needs further investigations.

While RON2 and RON5 are essential for tachyzoites, RON8 and RON4 are dispensable. Nevertheless, RON8 depletion led to a 70% reduction of invasion and a failure to close the PVM properly after successful invasion events. This invasion defect translates to radically impaired virulence in infected mice. Here we showed that RON4 L1 is not essential in vitro and the absence of RON4 L1 had no visible impact on the formation of the MJ or the vacuole. Accordingly, the RON4 L1 knock-out parasites we generated were not affected in the lytic cycle and invaded fibroblast cells as efficiently as wild-type parasites. Nevertheless we cannot exclude a potential compensatory effect through the up-regulation of other(s) gene(s), as it has been observed for RON2 and AMA1.

A delay of four to five days was observed compared to infection with parental parasites; this small but consistent delay was abolished by complementation with an exogenous RON4 L1 copy. It is not yet clear if the attenuation of Figure 5. Deletions of RON4 L1 does not affect the expression and localization of the RON2/RON4/RON5/RON8 complex. (a) Scheme representing the approach used to generate a straight knock-out for RON4 L1 in Δku80 HA+RON4 L1 strain. Two protospacers (pU6-RON4 L1 KO-1 and -2; in purple) were designed to induce a double strand break in the 5′UTR and 3′UTR of the RON4 L1 gene. A PCR amplifying the DHFR cassette flanked with 30 bp homology sequences of the 5′ and 3′UTR of RON4 L1 was used as a donor DNA fragment for DNA repair, inducing the removal of the entire RON4 L1 gene. Primers used to amplify RON4 L1, I3′ and I3′ fragments and the sizes of the PCR products are indicated. (b) PCR verification of DHFR integration at RON4 L1 locus. (c) Western blot using anti-HA antibodies shows the depletion of RON4 L1 in the KO-RON4 L1 strain. ROP5: loading control. (d) IFA on invading KO-RON4 L1 parasites using anti-ROP1, anti-HA, anti-RON2-4, anti-RON5, anti-RON4 and anti-RON4 antibodies. RON4 L1 labelling is lost in KO-RON4 L1 parasites while the other members of the RON complex are still present at the MJ. Scale bar, 2 µm. (e) Western blot analysis using anti-RON2-3, anti-RON5, anti-RON8 and rabbit anti-RON4 antibodies on HA + RON4 L1 parental line and KO-RON4 L1 mutant reveal normal expression of RON2, RON4, RON5 and RON8. Full-length blots and gels are presented in Supplementary Fig. 6.
virulence observed in vivo can be directly linked to a defect in invasion that was not discernible in our in vitro fibroblast assay. For instance, a specific role for RON4L1 during invasion of a peculiar cell type cannot be excluded. In the mouse model, slight alterations in parasite tropism, tissue migration, and invasion success rates can have potential greater consequences on parasite fitness. Transcriptomic and proteomic data sets in ToxoDB show that, in contrast to RON2, RON4, RON5 and RON8, RON4L1 is also expressed in the cat enteroprothelial stage. This could suggest also a role for RON4L1 in invasion of merozoites in the intestine.

In contrast to other Apicomplexa, Coccidia such as Toxoplasma and Neospora have very broad host specificity. The expression of additional RON proteins like RON8 and RON4L1 might illustrate an enriched MJ proteins repertoire in these parasites that would support their ability to invade a large variety of cell types.

Methods

Ethics statement. All animal work was conducted in strict accordance with the AAALAC (Association for Assessment and Accreditation of Laboratory Animal Care International) guidelines and the guide of animal care use book (Guide, NRC 2011). All mice protocols were approved by the Institutional Animal Care and Utilization Committee (IACUC) of the American University of Beirut (IACUC Permit Number IACUC#14-3-295). All animals were housed in specific pathogen-free facilities. Humane endpoints were used as requested by the AUB IACUC according to AAALAC (Association for Assessment and Accreditation of Laboratory Animal Care International) guidelines and guide of animal care use book (Guide, NRC 2011). Mice were sacrificed for any of the following reasons: 1) impaired mobility (the inability to reach food and water); 2) inability to remain upright; 3) clinical dehydration and/or prolonged decreased food intake; 4) weight loss of 15–20%; 5) self-mutilation; 6) lack of grooming behavior/rough/unkept hair coat for more than 48 hours; 7) significant abdominal distension; 8) unconsciousness with no response to external stimuli. Animals were deeply anesthetized before cervical dislocation. Eye pricks were done following deep anesthesia with isoflurane and all efforts were made to minimize suffering.

Parasite culture. All T. gondii tachyzoites were passaged in human foreskin fibroblasts (HFFs) (American Type Culture Collection-CRL 1634) or Vero cells (American Type culture Collection CCL 81) grown in Dulbecco’s modified essential medium (Gibco-BRL), supplemented with 5% fetal calf serum and 2 mM glutamine. Tachyzoites of the T. gondii RH strain deleted for ku80 gene (Δku80) were used throughout the study.

Figure 6. Phenotypic consequences of RON4L1 disruption. (a) Plaque assay of HA3-RON4L1 and KO-RON4L1 parasites. Parasites were added on HFF monolayer for 7 days and the size of lysis plaques was measured. AU: arbitrary units. Values are the mean standard error of the mean plaque area (20 plaques were measured in each condition) from one representative experiment out of two. No defect of the lytic cycle has been observed in the KO-RON4L1 mutant using a two-tailed t-test. (b) Invasion assay for 5 minutes in HFF cells of HA3-RON4L1 and KO-RON4L1 parasites. Values represent means ± SD, n = 3, from a representative experiment out of 2 independent assays. No defect of invasion was observed in vitro for the KO-RON4L1 strain using a two-tailed t-test. (c) In vivo virulence of Δku80, KO-RON4L1 and cpt-RON4L1 parasites in a mouse model. n = 5. Logrank tests show a significant difference between WT and KO-RON4L1 (P = 0.031) and between KO-RON4L1 and Cpt-RON4L1 (P = 0.0016), while no difference between WT and Cpt-RON4L1 was observed. Representative data out of 2 experiments.
Cloning of DNA constructs. Excepted when notified, all PCR amplifications were performed with the Phusion polymerase (NEB Biolabs) and the primers are listed in Supplementary Table S1.

To tag RON4Δ1 at the C terminal end of Δku80 parasites, we produced a pLIC-RON4Δ1 plasmid based on pLIC-DHFR-HA3. The 3’ end of RON4Δ1 gene was amplified with primers P11/P12, cloned in frame with the triple hemagglutinin tag in pLIC-DHFR-HA3 vector and linearized by XhoI prior to transfection. Single homologous recombination at the endogenous locus allowed the endogenous tagging of RON4Δ1.

To tag RON4Δ1 at the N terminal end just after the signal peptide, we used the CRISPR/Cas9 strategy. We designed a protospacer that would recruits Cas9 to cut 21 bases pairs downstream the end of the sequence signal codon and a donor DNA to insert triple HA by double homologous recombination. The sequence of the donor DNA is provided in Fig. S2. The protospacer sequence GACAATGCCGCCACGTGTGA was cloned by annealing primers P13 and P14 and cloning BsaI site of vector pU6-Cas9-YFP (gift of B. Striepen, UGA). The resulting plasmid pU6-RON4Δ1 Tag contains a Cas9-YFP fusion allowing selection of fluorescent parasites by FACS. The template sequence containing the HA3 sequence was generated by IDT services and TOPO-cloned. A 442 bases pair PCR product corresponding to the template was amplified with KOD polymerase (Novagen) using primers P15/P16 and co-transfected with pU6-RON4Δ1 Tag in Δku80 or KD-RON4 strains.

A CRISPR/Cas9 strategy was set up to generate a knock-out of the gene RON4Δ1. A fragment donor corresponding to the DHFR resistance cassette flanked by 30 bp homology arms of RON4Δ1 gene was amplified with KOD polymerase (Novagen) using primers P17 and P18. Two pU6-Cas9-YFP vectors were constructed. pU6-RON4Δ1KO-1 plasmid contains a protospacer targeting 11 bp before the start codon and pU6-RON4Δ1KO-2 plasmid targets 13 bp before the stop codon. pU6-RON4Δ1KO-1 and 2 plasmids were generated by annealing primers P19 and P20 or P21 and P22, respectively. The PCR product corresponding to the donor fragment and the two pU6-RON4Δ1KO plasmids were co-transfected in Δku80 HA3-RON4Δ1 strain allowing the removal of the entire RON4Δ1 gDNA of 22.2kb.

Completion of the KO-RON4Δ1 has been done through the integration of the cosmid PSBM65 (ToxoDB), which encompasses the entire gDNA of RON4Δ1 and contains a bleomycin cassette for selection. It was randomly inserted into the genome. Clones were obtained after phleomycin selection.

Immunoblots. Proteins from freshly egressed tachyzoites were resuspended into SDS buffer, separated on 10% SDS PAGE and transferred to nitrocellulose membranes. Primary antibodies used are listed in Supplementary Table S2 and diluted in 5% non-fat dry milk in TNT buffer (140 mM NaCl, 15 mM Tris, 0.05% Tween20). After three washes with TNT buffer, nitrocellulose membranes were incubated with alkaline phosphatase conjugated secondary antibodies and revealed with 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT; Promega).

Immunofluorescence microscopy. IFAs on intracellular parasites on HFFs cells were conducted as described previously30. Note that RON labelling in the rhoptries have been performed after formaldehyde 4% fixation, followed by 0, 1% saponin permeabilization. The antibodies used and their dilution for IFA are listed in Supplementary Table S2. Samples were observed with a Zeiss Axioimager epifluorescence microscope. Images were acquired with a Zeiss Axiocam MRm CCD camera driven at the ‘Montpellier resources imagerie’ facility and processed using Zeiss Zen software. Adjustments for brightness and contrast were applied uniformly on the entire image.

Transfection and selection of transformants. 20 × 10^6 T. gondii tachyzoites were transfected by electroporation at 2.02 kV, 50 Ω and 25 μF using an Electro Cell Manipulator 630 (BTX) with 30 μg of plasmid DNA as described previously30. 30 μg of CRISPR/Cas9 plasmids plus 5 μg of PCR products using the KOD polymerase were used to transfect parasites. Recombinant parasites were selected by addition of pyrithymethane at 2 μM for pLIC-RON4Δ1 and pKO-RON4Δ1 vectors, and 30 μg/ml of phleomycin for complementation. For N-terminal tagging of RON4Δ1, parasites transiently expressing cas9-YFP-fluorescence were sorted by FACS two days after transfection and cloned into a 96-well plate. For each transfection, clones were isolated by limiting dilution cloning and screened by PCR for correct DNA integration.

Plaque assays and invasion assays. Plaque assays and invasion assays were performed as described previously31. Independent invasion assays were performed three times in which at least 20 fields were quantified per coverslip (n = 3).

Co-immunoprecipitation. Parasites were solubilized in lysis buffer (1% NP40, 50 mM Tris-HCL pH8, 150 mM NaCl, 4 mM EDTA and protease inhibitor) and immunosorption procedures were done using anti-HA antibodies as described previously32. After overnight incubation of the lysate on beads, HA-beads are washed 5 times in wash buffer (30 mM Tris pH8, 1 M NaCl and 0.5% NP40). Elution from beads was performed during 5 min at 95 °C with SDS-PAGE sample buffer. Western blots were performed on the eluates using rabbit anti-RON2-3, rabbit anti-RON4, rat anti-RON5, rat anti-AMA1 and rat anti-HA antibodies. The antibodies dilutions used for western blot are described in Supplementary Table S2.

Glass beads antibody loading. Loading of antibodies was performed using glass-beads as originally described32. Acid-washed 150–212 μm glass beads (Sigma) were washed 3 times with distilled water. 0.1 mg of beads were then resuspended in 300 μl of the appropriate medium containing commercial anti-HA antibodies (Covalab) diluted at 1/30. HFF cultures growing on coverslips in a 24 wells-plate were washed twice with Hanks’ Balanced Salt Solution (HBSS) before the antibodies-beads solution was put into each well. The beads were rolled onto the coverslip by tilting the plate ∼10 times, until evenly distributed over its surface. The coverslip was then...
transferred to another well where it was washed 3 times with HBSS and returned to DMEM culture medium and allowed to recover at 37 °C and 5% CO2 for 30 minutes. Invasion assays were then carried out by allowing T. gondii tachyzoites to sediment on the HFF for 20 minutes at 4 °C and subsequently warming them during 2.5 min at 37 °C to trigger invasion. Invasion was stopped and cells were fixed by adding an excess volume of 4% PAF in HBSS. The extracellular portion of the tachyzoites was labelled with rabbit SAG1. Parasites and cells were then permeabilized with 0.1% saponin and incubated with secondary antibodies.

**Survival in vivo.** 100 tachyzoites freshly harvested from cell culture were inoculated by intra-peritoneal (i.p.) injection in 8–10 week-old female BALB/c mice (Jackson laboratories). Simultaneously to injection, parasites infectivity was evaluated by plaque assay. 7 days post-infection, seropositivity against *Toxoplasma* infection was tested. Survival experiments were done on groups of 5 mice per parasite cell line. Survival experiments were repeated independently twice. Mice survival was checked on a daily basis until their death, endpoint of all experiments. Data were represented as Kaplan and Meier plots using Prism software (Graphpad).

**Statistical analysis.** All results are presented as mean values SEM. For invasion and plaque assays, one representative experiment is shown and two-tailed t-test has been used to determine significance. For *in vivo* experiments, levels of significance were determined with the Logrank test using GraphPad.

**Data availability.** All relevant data are included in the paper or the Supplementary Information.

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**Author Contributions**

A.G. and M.L. designed the study; A.G. performed all the study related to RON4, and its phenotypic characterization *in vitro*. A.G. and S.B. performed the topology analysis of RON4, H.E.H. performed *in vivo* experiments. D.P.V. performed immuopurifications and immunofluorescences. A.G. and M.L. analysed the data and wrote the manuscript.

**Additional Information**

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