C-Mannosylation of Toxoplasma gondii proteins promotes attachment to host cells and parasite virulence

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**Running title:** Importance of protein C-mannosylation for *Toxoplasma gondii*

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**ABSTRACT**

C-Mannosylation is a common modification of thrombospondin type 1 repeats present in metazoans and recently identified also in apicomplexan parasites. This glycosylation is mediated by enzymes of the DPY19 family that transfer α-mannoses to tryptophan residues in the sequence WX₂WX₂C, which is part of the structurally essential tryptophan ladder. Here, deletion of the *dpy19* gene in the parasite *Toxoplasma gondii* abolished C-mannosyltransferase activity and reduced levels of the micronemal protein MIC2. The loss of C-mannosyltransferase activity was associated with weakened parasite adhesion to host cells and with reduced parasite motility, host cell invasion, and parasite egress. Interestingly, the C-mannosyltransferase–deficient Δ*dpy19* parasites were strongly attenuated in virulence and induced protective immunity in mice. This parasite attenuation could not simply be explained by the decreased MIC2 level and strongly suggests that absence of C-mannosyltransferase activity leads to insufficient level of additional proteins. In summary, our results indicate that *T. gondii* C-mannosyltransferase DPY19 is not essential for parasite survival, but is important for adhesion, motility and virulence.
for parasite replication (2,4). Parasites egress the invaded cell by rupturing both the PV and host cell plasma membranes prior to invading neighboring cells. The parasite lytic cycle relies on the sequential and regulated secretion of proteins from specialized apical organelles called micronemes and rhoptries (5,6).

Micronemal proteins (MICs) typically contain evolutionary conserved modular domains such as epidermal growth factor like, PAN/Apple or thrombospondin type 1 repeats (TSRs) that mediate protein-protein and protein-carbohydrate interactions (7,8). These interactions are involved in the formation of MIC complexes and enable attachment of the parasite to host cells (8). Several MICs (e.g. MIC2, MIC6 or MIC8) also interact via their cytoplasmic domain with the parasite submembrane actomyosin system and thus bridge the parasite cytoskeleton and the host cell. The actomyosin system is part of a multi-protein complex known as the glideosome that ensures translocation of MIC complexes engaged to host cell receptors from the apical pole of the parasite toward the posterior pole. This rearward translocation enables gliding motility of the parasite and is required for parasite migration through tissues, invasion of host cells and egress from infected cells (2,3).

Microneme exocytosis, a prerequisite to parasite attachment and gliding motility, follows a rise of the cytosolic calcium concentration. The release of calcium from the endoplasmic reticulum (ER) and other internal stores is typically triggered by a signaling cascade in response to a low extracellular potassium level, but can be artificially induced through ethanol exposure (6,9,10). After their release onto the parasite surface, MIC proteins often undergo proteolytic processing. The microneme subtilisin protease SUB1 has been shown to trim several MIC proteins such as MIC2-M2AP complex is essential for its proper trafficking to the micronemes (24,27). Formation of the sixth TSR of MIC2 has also been shown to associate with M2AP in the ER to form a heterohexamer (24,27). Formation of the MIC2-M2AP complex is essential for its proper trafficking to the micronemes (22,28,29). In contrast to MIC2, genetic ablation of SPATR did not significantly reduce adhesion or gliding motility but decreased host cell invasion (20). The function of other TSR-containing proteins remains to be established.

Two distinct types of glycosylation, α-O-fucosylation and α-C-mannosylation, have been shown to modify TSRs in metazoans and have been recently described in Plasmodium falciparum and P. yoelii thrombospondin-related anonymous protein (TRAP), P. yoelii circumsporozoite protein (CSP) and T. gondii MIC2 (30-35). α-O-fucosylation of folded TSRs is catalysed by the protein O-fucosyltransferase POFUT2 that acts on serine or threonine residues in the consensus sequence CX2,3(S/T)CX3,G (which comprises conserved cysteines) (31,32,36-39). The β1,3-glucosyltransferase B3GLCT may then add a terminal glucose to generate the disaccharide Glcβ1-3Fucα-O-Ser/Thr (40-42). Protein-O-fucosylation occurs in the ER on folded
proteins and has been shown to stabilize and promote trafficking of some TSR-containing proteins in metazoans, P. falciparum and Toxoplasma gondii (31,38,43). The O-fucosylation motif is directly preceded by a WX2WX3WX2C or WX2WX3C sequence that may carry α-mannose residues linked via a carbon-carbon bond to the tryptophan residues. The latter are part of a tryptophan-arginine ladder that plays a central role in the TSR fold. The mannose residues are transferred from dolichol phosphate mannose by specific C-mannosyltransferases of the DPY19 family (44,45). As all other glycosyltransferases that use dolichol-phosphate activated substrate, DPY19 proteins are rather large multi-pass membrane glycosyltransferases localized in the ER (45). The family name originates from the dumpy phenotype observed in C. elegans worm carrying loss of function mutation in the dpy-19 gene. The phenotype associates with a defect in neuronal migration and is similar to the phenotype caused by deficiency in the C-mannosylated protein MIG-21 (45). In mammals, at least two DPY19 proteins (DPY19L1 and DPY19L3) are required for C-mannosylation of proteins having a WX2WX2 or WX2WX3C motif, including TSRs and type I cytokine receptors (44). We have recently demonstrated that T. gondii and P. falciparum DPY19 are C-mannosyltransferases acting on microneme proteins of the TRAP/MIC2 family (30) and investigated here the importance of C-mannosylation for motility, invasion and virulence of T. gondii by targeted gene deletion.

Results

Toxoplasma gondii protein C-mannosylation is abrogated upon dpy19 deletion

To assess the importance of C-mannosylation in T. gondii, the dpy19 gene was replaced by a cassette encoding a pyrithymethane resistant dihydrofolate reductase-thymidylate synthase (DHFR-TS) via homologous recombination in the RHΔhxgprt strain (herein designated as wild type) (Fig. 1A). Gene replacement was facilitated by a CRISPR/Cas9 genome editing using two guide RNAs (gRNAs) targeting the regions immediately 5’- and 3’- of dpy19. The resulting Δdpy19 strain was selected using pyrithymethane and single clones were isolated by limiting dilution. Three clones were selected and analyzed in this study. Replacement of dpy19 was confirmed by genomic PCR analyses (Fig. 1B). To functionally rescue the Δdpy19 mutant, a plasmid encoding myc-tagged DPY19 and the selection marker chloramphenicol acetyltransferase (CAT) was transfected in the Δdpy19 strain. The complemented strain named Δdpy19comp was selected with chloramphenicol and was shown to express MycDPY19 by Western blot (Fig. 1C). By immunofluorescence analysis, MycDPY19 co-localized with the transiently expressed Tyr-tagged acetyl-CoA transporter AT1, previously shown to be in the ER membrane (46) (Fig. 1D).

To confirm the absence of functional C-mannosyltransferase, enzymatic assays were performed with microsomal fractions isolated from wild type, Δdpy19 and Δdpy19comp tachyzoites (Fig. 1E). The microsomal preparations were incubated with radioactive GDP-Man (precursor of the dolichol-phosphate mannose donor substrate) and the acceptor peptide WAEWGEC. After incubation of the mixture, the peptide was extracted, purified by reverse phase chromatography and the associated radioactivity was measured. The peptide WAKW, which is an acceptor of mammalian DPY19 proteins but not of T. gondii DPY19, was used as control (30). As expected, C-mannosylation of the peptide WAEWGEC but not WAKW was observed in microsomes from the wild type strain. Importantly, this enzymatic activity was absent in the Δdpy19 strain indicating that DPY19 is the only C-mannosyltransferase present in T. gondii, and was restored in the Δdpy19comp strain (Fig. 1E).

The Δdpy19 strain presents a defect in parasite adhesion and motility

Multiple rounds of lytic cycles over 7 days of culture resulted in plaque formation within host cell monolayers. C-mannosylation deficient parasites clearly formed smaller plaques in comparison to the parental and Δdpy19comp strains (Fig. 1F). A series of assays was thus carried out to define which steps of the lytic cycle were affected. We first examined the rate of replication by counting the number of parasites per vacuole, 24 h post invasion. The Δdpy19 mutant did not display any defect in intracellular growth as indicated by the similar number of Δdpy19 or wild type parasites per vacuole (Fig. 1G). In contrast,
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Both the ability of Δdpy19 parasites to invade host cells and to egress from these cells was significantly impaired (Fig. 1H and I). Merely 25.0 (± 6.1) % of Δdpy19 parasites were found to invade cells as compared to 57.0 (± 4.8) % for wild type parasites representing a reduction of the invasion rate by approximately 44% (Fig. 1H). In contrast, invasion of cells by the Δdpy19comp and wild type strains was comparable (Fig. 1H). When infected cells were treated with the Ca²⁺ ionophore A23187 to induce parasite egress (47), 39.0 (±16.5) % of Δdpy19 parasites containing vacuoles were able to rupture versus 85.5 ± (2.4) % for the parental strain (Fig. 1I).

Since host cell invasion and parasite egress are intimately connected to parasite attachment and gliding motility, we investigated these processes in Δdpy19 parasites and the parental strain. In vitro gliding assay was performed using live-video microscopy to dissect the twirling, circular and helical movements previously described (48). Only 19.3 (± 3.5) % of the Δdpy19 parasites were motile compared to 58.3 (± 9.3) % of wild type parasites (Fig. 1J). The impaired motility of Δdpy19 parasites was associated to a defect in attachment. Indeed, using a standard immunostaining assay, we observed that only 29.8 (± 3.7) % of Δdpy19 parasites attached to cultured fibroblasts compared to the wild type strain (Fig. 1K).

**Loss of C-mannosylation leads to reduced MIC2 cellular level**

To date, the abundant micronemal adhesin MIC2 is the only protein that has been shown to be C-mannosylated in *T. gondii* (30-32). In an attempt to demonstrate absence of MIC2 C-mannosylation in Δdpy19, glycopeptides obtained by in gel digestion with trypsin and AspN were analyzed by liquid chromatography coupled to tandem mass spectrometry (nanoUPLC-MS/MS). As expected, the TSR5 peptide DERPGEAWEGCSVTGC was C-mannosylated and O-fucosylated in samples obtained from wild type parasites (Fig. S1). In samples obtained from Δdpy19 parasites, this peptide (with or without C-mannosylation and/or O-fucosylation) was not observed, although the detection of other MIC2 peptides confirmed the presence of this protein (Fig. S1). Other peptides containing C-mannosylation sites were not observed. These data indirectly support absence of C-mannosylation in the Δdpy19 strain.

Given the presumed role of C-mannosylation in protein folding and/or stabilization (44,45,49,50), we examined whether the cellular level of MIC2 was influenced by the loss of C-mannosylation. Lysates of wild type, Δdpy19 and Δdpy19comp tachyzoites were analyzed by Western blot using an anti-MIC2 antibody and an anti-tubulin antibody to normalize loading in all lanes (Fig. 2A). MIC2 carries at least 9 mannose residues (~1.5 kDa) (31,32,39), whose absence in the Δdpy19 strain is supported by the slightly faster migration of MIC2 when compared to the parental strain (Fig. 2A). Moreover, a decrease of approximately 50 % (46 ±15 %) in MIC2 cellular level was observed in the Δdpy19 mutant compared to the wild type (Fig. 2A). As expected, the migration and level of MIC2 were restored in the complemented strain Δdpy19comp.

Since the level and trafficking of MIC2 was previously shown to depend on association with M2AP (22,28), the interaction of MIC2 with M2AP was analyzed by immunoprecipitation using an anti-M2AP antibody and detection with an anti-MIC2 antibody (Fig. 2B). MIC2 efficiently co-immunoprecipitated with M2AP, indicating that the interaction of these two proteins was not dependent on C-mannosylation (Fig. 2B). Moreover, a decrease of the proteolytic maturation of M2AP from its proform to mature form was observed in the Δdpy19 mutant, which is in perfect agreement with a reduction of MIC2 cellular level (Fig. 2B) (22). Consistent with the formation of a complex, MIC2 and M2AP localized at the apical region of parasites, corresponding to the micronemes, in the Δdpy19 and wild type strains (Fig. 2C).

Finally, to determine if secretion and proteolytic processing of micronemal proteins was influenced by absence of C-mannosylation, ethanol was added to freshly egressed tachyzoites to trigger microneme secretion and the excretory-secretory antigens (ESA) were analyzed by Western blot. Catalase and the dense granule protein 1 (GRA1) were used as loading control for cellular and constitutively secreted proteins, respectively. Proteolytic processing and secretion of MIC6 and AMA1, which do not contain any TSR or C-mannosylation motif, was similar in all strains. In line with the
reduced cellular level of MIC2, the relative amount of secreted MIC2, which migrated as a doublet due to differential trimming of its N-terminus, was reduced in the \( \Delta \text{dpy19} \) strain compared to the parental and complemented strain.

**Deletion of dpy19 results in loss of virulence in mice and protection against a subsequent parasite challenge**

To address the impact of protein C-mannosylation on parasite virulence, 5 mice were inoculated intraperitoneally with 50 parasites of the wild type, \( \Delta \text{dpy19} \) or \( \Delta \text{dpy19} \text{comp} \) strain. After 8-9 days, mice that had been infected with the wild type or complemented strain were sacrificed due to severe symptoms. In contrast, mice infected with the \( \Delta \text{dpy19} \) mutant showed no symptoms, although seroconversion confirmed infection. To determine whether these mice were protected against a subsequent challenge, 100,000 wild type parasites were inoculated on day 21 after the first infection. All challenged mice survived, which indicated that \( \Delta \text{dpy19} \) had induced protective immunity (Fig. 3).

**In silico prediction of C-mannosylated proteins in T. gondii**

The in vitro C-mannosyltransferase assays presented in figure 1C confirmed that \( T. gondii \) DPY19 modifies the WAEGWEC peptide but, in contrast to metazoans C-mannosyltransferases, does not act on the shorter WAKW peptide (Fig. 1E) (30,51). These data and the few C-mannosylation sites described in \( T. gondii \), \( P. falciparum \) and \( P. yoelii \) proteins (30-35,39) strongly suggest that apicomplexan C-mannosyltransferases recognize WX\(_2\)WX\(_2\)C motifs, commonly found in TSRs. A protein Blast search identified 37 \( T. gondii \) proteins with a WX\(_2\)WX\(_2\)C sequence. A previous study of experimentally verified C-mannosylation sites indicated that metazoan C-mannosyltransferases have a strong preference for WX\(_2\)W or WX\(_2\)C motifs with a serine, alanine, glycine or threonine following the tryptophan residue (52). The few C-mannosylation sites described in apicomplexan proteins to date suggest that the parasite DPY19 enzymes have a similar specificity (30-35,39). Table S1 and S2 present the 14 \( T. gondii \) proteins containing at least one WX\(^*\)WX\(^*\)XC motif, in which the amino acids X\(^*\) are a serine, alanine, glycine or threonine. Besides the WX\(_2\)WX\(_2\)C motif(s), conserved cysteine, arginine and glycine residues are present in the vast majority of these proteins and indicate their relation to the TSR superfamily (53). These proteins represent potential substrates of \( T. gondii \) DPY19. This list is, however, potentially not exhaustive given that slight alterations of the WX\(_2\)WX\(_2\)C recognition motif can be tolerated, as seen by the C-mannosylation pattern of MIC2 (Table S2) (39), and information about the exact specificity of C-mannosyltransferases is still limited.

**Discussion**

In apicomplexans, like in metazoans, little is known about the extent and function of protein C-mannosylation. In this study, we analyzed the importance of this protein glycosylation for the lytic cycle and virulence of \( T. gondii \) tachyzoites. Deletion of \( T. gondii \) dpy19 gene was successful, revealing that protein C-mannosylation is not essential for parasite survival. Similarly, DPY19 was lately shown to be dispensable for \( P. falciparum \) asexual blood stages viability (54). Genetic screens predicted, however, that DPY19 confers fitness to \( T. gondii \) tachyzoites and \( P. falciparum \) asexual stages (53,55). In agreement, DPY19 was shown to play important functions during the lytic cycle of \( Toxoplasma \), as seen by the small plaques formed by the dpy19 deficient strain. Importantly, the plaque size was fully restored by re-expression of the C-mannosyltransferase. Detailed phenotyping revealed that absence of protein C-mannosylation severely compromised parasite adhesion, which as expected was associated with reduced motility, invasion and egress.

The micronemal protein MIC2 secreted by tachyzoites has recently been shown to be C-mannosylated and O-fucosylated (30-32). Absence of MIC2 C-mannosylation in the \( \Delta \text{dpy19} \) strain was suggested by the faster migration of MIC2 in SDS-PAGE and absence of C-mannosyltransferase activity in the knockout strain. Importantly, the cellular level of MIC2 was decreased by approximately 50% in the \( \Delta \text{dpy19} \) strain. The formation of the MIC2-M2AP complex involves the 6\(^{th}\) TSR of MIC2 and the modified galectin domain of M2AP (24,27) and is determinant for the cellular level, trafficking and secretion of both proteins (22,28). The reduction of MIC2 level in the \( \Delta \text{dpy19} \) strain was, however, not due to the
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abundance of interaction with M2AP since the two micronemal proteins co-immunoprecipitated and were co-localized at the apical pole of the parasite. In line with this result, it was recently demonstrated that the 6\textsuperscript{th} TSR of MIC2 is not C-mannosylated and not O-fucosylated in Toxoplasma tachyzoites (24,27,31,32). As expected, deletion of the O-fucosyltransferase POFUT2 did not affect the MIC2-M2AP complex formation either (31,32). Together, these data confirm that association of MIC2 and its escort protein M2AP is mediated by protein-protein interaction (27).

In T. gondii, like in metazoan, the C-mannosyltransferase DPY19 localized to the ER, as is the case for other known glycosyltransferases that use dolichol-phosphate linked donor substrate. C-mannosylation might be co-translational, since folded proteins have been shown to be poor acceptor substrates \textit{in vitro} (51). Indeed, expression of TSR-containing proteins in C-mannosylation deficient cells is often associated with poor yield, suggesting that this glycosylation process assists protein folding and/or contributes to protein stability (44,45,49,50,56,57). The involvement of C-mannosylation in TSR folding and stability was lately confirmed for the netrin receptor UNC5 (Sheberbakova et al., unpublished). However, the requirement for C-mannosylation seems to vary from protein to protein. MIC2 contains 6 TSRs and is modified with at least 9 C-mannose residues (31,32,39). In the \(\Delta dpy19\) strain, this protein might be partially misfolded and degraded by the ERAD pathway leading to the observed decrease in MIC2 level. The remaining MIC2 is presumably folded, associates with M2AP and is properly trafficked to the micronemes before being secreted.

In the absence of MIC2, the parasite ability to attach to host cells was impaired, with severe consequences on motility and host cell invasion (22,23). In \(\Delta dpy19\) parasites, the observed attachment and invasion defects were less pronounced and resemble the defects reported for a mutant deficient in the protein \(O\)-fucosyltransferase POFUT2 (31). Note that one of the described \(\Delta pofut2\) mutants presented significantly lower MIC2 level and an invasion defect (31), whereas the second displayed no substantial changes in attachment or MIC2 abundance (32). A third \(\Delta dpy19\) mutant displaying a small plaque phenotype has been recently generated and might help resolve this controversy (42).

The phenotype of the \(\Delta dpy19\) clearly differs from the phenotype of the MIC2 deficient strain \(\Delta mic2\) since \(T. gondii\) preserves its virulence in absence of MIC2 (23), whereas the lack of C-mannosyltransferase activity leads to a strong attenuation of virulence. A study involving simultaneous disruption of \(mic1\) and \(mic3\) has previously demonstrated that microneme proteins have synergetic roles not only in adhesion and invasion but also in virulence (58). The different virulence phenotype observed in \(\Delta dpy19\) and \(\Delta mic2\) strongly suggests that, in addition to MIC2, other proteins are impacted by loss of C-mannosyltransferase activity. Analysis of C-mannosylated peptides by tandem mass spectrometry is the method of choice to identify C-mannosylated proteins. However, this method is often applied to isolated proteins and requires manual annotation of the potential glycopeptides spectra. Recently, complex protein lysates from Plasmodium sporozoites or Toxoplasma tachyzoites have been analyzed, but only the abundant surface proteins TRAP, CSP (in \(P. yoelii\)) and MIC2 were identified as C-mannosylated proteins (30-35,39). Based on our limited knowledge of C-mannosyltransferases specificity, the micronemal proteins MIC12, MIC14, MIC15 and MIC16, whose functions are currently unknown, as well as several uncharacterized proteins were identified here as candidate C-mannosylated proteins. Further studies will be needed to confirm C-mannosylation of additional Toxoplasma proteins and assess their role in parasite biology and virulence.

Materials and Methods
Parasite culture
\(T. gondii\) tachyzoites of RH \(\Delta ku80\Delta hsgprt\) strain (59), herein referred as wild type or parental strain, \(\Delta dpy19\) and \(\Delta dpy19\) comp (this study) were maintained by serial passage on monolayers of Human foreskin fibroblasts (HFF; ATCC® SCRC-1041™) cultured in Dulbecco’s modified Eagle’s medium (DMEM; Biochrom, Germany) supplemented with 5% (v/v) heat inactivated foetal bovine serum (iFBS, Biochrom, Germany) at 37 °C and 5% CO\(_2\).
Deletion of *T. gondii* *dpy19*

All primers used are listed in Table S3. A DHFR-TS (dihydrofolate reductase-thymidylate synthase) selection cassette (including promoter and terminator regions) was amplified from plasmid p2854 (60) using the primers AFA96/AFA97, which each contains 30 nucleotides homologous to the 5'- or 3'-untranslated region of *dpy19*. Additionally, plasmid pU6-dpy19, which encodes a single guide RNA (sgRNA) targeting the region immediately upstream of the start codon (gcagactcgctctcggc) and a sgRNA targeting the stop codon downstream (agttaatcttccttctccggc) was generated and used to enhance the insertion of the DHFR-TS cassette. To generate pU6-dpy19, *T. gondii* U6 termination sequence and promoter were amplified from plasmid p2sgRNA with primers AFA102/AFA103, which each includes a sgRNA sequence. The amplicon was then cloned in the Bsal restriction site of pU6-Universal (between U6 promoter and terminator) (Addgene plasmid #52694) (61). The resulting plasmid encodes 2 sgRNAs targeting the stop codon downstream (agttaatcttccttctccggc) was generated and used to enhance the insertion of the DHFR-TS cassette. To generate pU6-dpy19, *T. gondii* U6 promoter and *S. pyogenes* Cas9 under the TUB1 promoter. Freshly egressed RH Δku80Δmxgp prt tachyzoites (~ 10^7 parasites) were pelleted at 1000 G for 5 min and washed with cytomix buffer. The pellet was resuspended in cytomix buffer containing 2 mM ATP, 5 mM glutathione, 20 μg of the plasmid pU6-dpy19 and 7 μg of the DHFR-TS selection cassette in a final volume of 800 μL. Electroporation was performed in a 4 mm cuvette with 2 pulses of 2 kV, 50 Ω, 25μF. Transformants were selected using 1 μM pyrimethamine. The clonal Δdpy19 line was isolated by performing serial dilutions in the presence of drug selection followed by verification by Western Blot and immunofluorescence assay using a mouse hybridoma supernatant anti-Myc (1:100).

Preparation of microsomal fractions and in vitro C-mannosyltransferase assays

Approximately 10^7 tachyzoites were harvested from lysed CHO cells deficient in DPY19L1, -L2, -L3 and -L4 and suspended in 2 mL ice cold lysis buffer (10 mM HEPES-Tris pH 7.4, 0.8 M sorbitol, 1 mM EDTA containing protease inhibitor cocktail (Roche)) and disrupted by nitrogen cavitation at 450 psi, two times, for 10 minutes, on ice. The homogenate was centrifuged at 1500 x g, for 10 minutes at 4°C. Supernatant was further centrifuged at 100 000 x g for one hour and the microsomal pellets were resuspended in 200 μL 10 mM MOPS (pH 7.5). Microsomal fractions were aliquoted and kept at -80°C.

C-mannosyltransferase assays were performed as previously described (30). Reactions contained microsomal fraction (10 μL representing ~35 μg total protein), 3.7 kBq GDP[^H]-Man (American Radiolabeled Chemicals), 2 μM GDP-Man, 100 mM MOPS (pH 7.5). Microsomal fractions were aliquoted and kept at -80°C.

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Complementation of the Δdpy19 strain

For complementation of Δdpy19, a plasmid coding for N-terminally Myc-tagged DPY19 and the selection marker chloramphenicol acetyltransferase (CAT) was generated. Therefore, *T. gondii dpy19* coding sequence was amplified from pcDNA3.1-dpy19 (30) using primers AFA114R and AFA115F and inserted in Nsil/PacI sites of pTub8MycGFPpMuyoAtaiTy-HXGPRT plasmid (62). The tubulin promoter and Myc-DPY19 were then amplified using AFA116F and AFA118R and inserted into the EcoRV/BglIII sites of the pCAT vector, which contained 300 and 380 nucleotides homologous to the 5'- and 3'-UTR of the uracil phosphoribosyltransferase (UPRT), resulting in p5'UPRT-CAT-pTub8-mycTgDPY19-3'UPRT.

Electroporation was performed as described above with 10 μg of pSAG1Cas9gfp-U6sgUPRT plasmid (63) and 40 μg of *NotI*KpnI linearized 5'UPRT-CAT-pTub8-mycTgDPY19-3'UPRT. Parasites were selected using 20 μM of chloramphenicol.

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The cartridges were washed 3 times with 3 mL 0.1% TFA and peptides were eluted with 4 mL of methanol. After evaporation of the methanol, 2 mL Luma Safe (Zinsser Analytic) were added and the samples were counted in a Beckman Coulter LS 6500.

**Plaque assay**
Freshly egressed parasites were inoculated on a confluent monolayer of HFF and incubated 7 days at 37 °C and 5% CO₂, after which HFF were washed once with PBS and fixed with 4% paraformaldehyde/0.05% glutaraldehyde for 10 minutes. Fixed HFF were stained with crystal violet solution (0.1%) and washed three times with PBS. Plaques were measured using Fiji software version 1.8.0_66. Mean values of three independent experiments ± SD were determined.

**Immunofluorescence assay (IFA)**
Parasite-infected HFF cells seeded on cover slips were fixed in 4% paraformaldehyde (PFA)/0.05% glutaraldehyde (GA) in PBS for 10 minutes. After fixation, cells were rinsed once with 0.1 M glycine in PBS. Cells were permeabilized with 0.2% Triton X-100 in PBS for 20 minutes and blocked in PBS with 2% BSA. Cells were incubated for 60 min with primary antibodies diluted in blocking buffer, washed, and incubated for 60 min with secondary antibodies IgG Alexa Fluor 488 and 568 (1:500, Molecular Probes). Slides were viewed on a Zeiss Epifluorescence microscope using 40x or 63x/1.4 oil objectives, imaged with an Axio Cam MRC (Zeiss) camera and analyzed with Zen 2012 (blue edition, version 6.1.7601) software or with a Zeiss microscope (LSM700, objective apochromat 63/1.4 oil).

**Intracellular growth assay**
Freshly egressed parasites were allowed to invade a monolayer of HFF at 37 °C and 5% CO₂ for 24 hours. After which, media was exchanged for pre-warmed, serum-free DMEM containing 3 μM A23187 in DMSO and incubated for 7 minutes at 37 °C. IFA was performed using mouse hybridoma supernatant anti-GRA3 (1:100) and rabbit polyclonal anti-IMC1 (1:1000). For each condition, 200 vacuoles were counted and the number of lysed vacuoles was scored. Mean values of triplicates from three independent experiments +/- SD were determined.

**Induced egress assay**
Freshly egressed parasites were allowed to invade a monolayer of HFF for 24 hours. After which, media was exchanged for pre-warmed, serum-free DMEM containing 3 μM A23187 in DMSO and incubated for 7 minutes at 37 °C. IFA was performed using mouse hybridoma supernatant anti-GRA3 (1:100) and rabbit polyclonal anti-IMC1 (1:1000). For each condition, 200 vacuoles were counted and the number of lysed vacuoles was scored. Mean values of triplicates from three independent experiments +/- SD were determined.

**In vitro gliding motility assay**
Gliding was monitored by video microscopy on a Nikon eclipse Ti inverted microscope. Freshly egressed RH Δku80Δhxgppt and Δdpy19 parasites were allowed to settle onto glass chamber slides (Ibidi) coated with 0.1% gelatin. Prior to imaging, 5-Benzyl-3-isopropyl-1H-pyrazolo[4,3-d]pyrimidin-7(6H)-one (BIPPO) was added to the medium at a final concentration of 5 μM and parasites were captured by time-lapse microscopy for 1 min in several areas of the chamber using a 63x Oil Plan Apochromat objective. One hundred parasites were used to score gliding behaviors (i.e., circular, helical or twirling) or no productive movement. Experiments have been done in triplicate.

**Attachment assay**
Attachment assay was conducted as described previously (23). Briefly, 10⁶ freshly egressed tachyzoites were inoculated on a confluent monolayer of HFF and incubated for 10 minutes at 37 °C and 5% CO₂, after which cells were washed 3 times with 3 mL 0.1% TFA and peptides were eluted with 4 mL of methanol. After evaporation of the methanol, 2 mL Luma Safe (Zinsser Analytic) were added and the samples were counted in a Beckman Coulter LS 6500.
were washed once with PBS. IFA was performed using mouse hybridoma supernatant anti-SAG1 (1:100) and total numbers of parasites within 15 fields of view (Objective 60x) were counted. Mean value of triplicates from three independent experiments +/- SD were determined.

**Cellular localization of DPY19**

*T. gondii at1* (TgME49_215940) was amplified from cDNA using the primers DSF5272/DSF5273 and inserted in EcoRI/NsiI sites of pTub8MycGFPrMycAtailTy-XGPRT plasmid (62) to generate the pTub8-AT1Ty-HXGPRT plasmid. Around 10 µg of the plasmid were then transiently transfected in the Δdpy19 comp strain. Immunofluorescence assay using anti-Myc (DPY19) and anti-Ty (AT1) antibodies was performed on the next day.

**Mass spectrometry analyses**

Wild type and Δdpy19 tachyzoites were disrupted in 500 mM Tris-HCl pH 8.5, 50 mM EDTA, 700 mM sucrose, 100 mM KCl, 1% SDS and 2% β-mercaptoethanol by sonification 8 x 30s on ice (-10^8 parasites/mL). One volume Roti-phenol pH 7.5-8.0 (Roth) was added to samples and mixed by inversion for 10 min at 4°C. The phenol phase was precipitated with 4 volumes of 0.1 M ammonium acetate in cold methanol at -20°C for at least 4 hours. The pellet recovered by centrifugation at 6000G for 10 min at 4°C was solubilized in Laemli buffer and proteins (equivalent to 5x10^7 parasites/lane) were separated on a 10% SDS-PAGE gel. The MIC2 containing bands were manually excised from Coomassie gel and treated as previously described (30). Briefly the proteins were reduced with 10 mM DTT, alkylated with 100 mM iodoacetamide and digested at 37°C with 0.1µM trypsin followed by digestion with 0.1 µg AspN (Promega). The resulting peptides were extracted from gel pieces with 50% acetonitrile containing 5% formic acid, followed by 75% acetonitrile containing 0.5% formic acid and finally 100% acetonitrile. Extracts were dried and dissolved in 2% acetonitrile containing 0.1% trifluoroacetic acid. A Waters nanoACQUITY-UPLC System equipped with an analytical column (Waters, BEH130 C18, 100 µm × 100 µm, 1.7 µm particle size) coupled online to an ESI-Q-TOF Ultima was used for analysis as previously described (44). Obtained spectra were explored with MassLynx V4.1Software (Waters). The theoretical mass of each peptide containing putative C-mannosylation sites was calculated with or without glycosylation (C-mannosylation and O-fucosylation). Extracted ion chromatograms were generated by ion counts for these masses (±0.1Da).

**Analysis of MIC2 cellular level by Western Blotting**

Tachyzoites were harvested, centrifuged at 1500G for 10 min, suspended at a concentration of 10^6 parasites/mL in Laemli buffer and disrupted by sonification 8 x 30s on ice. 1% β-mercaptoethanol was added and samples were heat at 95°C for 10 min. Protein were separated by SDS-PAGE on a 12% acrylamide gel and blotted onto nitrocellulose membrane. The membrane was stained with the mouse anti-MIC2 6D10 hybridoma supernatant (1:100) or mouse anti-α-tubulin (1:800) (Developmental Studies Hybridoma Bank) followed by an anti-mouse IRDye800CW (1:20 000) (LiCor Biosciences) in Odyssey buffer (LiCor Biosciences). Labelled membranes were detected using LiCor Odyssey infrared imager 1060 v2.1.12, and the images processed with Image Studio v4.0.21.

**MIC2-M2AP co-immunoprecipitation**

HFF monolayers from a 6 cm² dish were infected with tachyzoites. 48 hours post-infection, extracellular and intracellular parasites were purified by passing the cell-parasite suspension two times through 27G needles (Braun), washed once with PBS, pelleted and resuspended in 0.5 mL of immunoprecipitation (IP) buffer (1% Triton X-100, 50 mM Tris-HCl pH 8.0, 150 mM NaCl, EDTA-free proteases inhibitor (Roche). Parasite suspension was freezeed and thawed five times, sonicated 3 x 10 seconds on ice and centrifuged 14,000 g for 30 minutes at 4 °C. Supernatant was incubated with a rabbit anti-M2AP antibody for 60 min at 4 °C on a rotating wheel. After incubation, 0.1 mL of Protein A-Sepharose TM CL-4B (GE Healthcare Life Sciences) beads was added to the suspension and sample was incubated for 1 hour at 4 °C on a rotating wheel. Complexes were washed 4 times in 1 mL of IP buffer with intermediate centrifugation at 1,500 g for 1 min, at 4 °C. The remaining pellet was suspended in protein loading buffer with 5% DTT, separated in a 12 % SDS polyacrylamide
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gels and transferred to a nitrocellulose membrane. Western-blots were processed using anti-MIC2 antibody for 60 min, washed and incubated with IRDye LiCor secondary antibody for another 60 min. Labelled membranes were detected using Li-Cor Odyssey infrared imager ODY 1060 v2.1.12, and the images processed with Image Studio v4.0.21.

Microneme secretion assay
Freshly egressed parasites were harvested, washed twice with pre-warmed intracellular buffer (5 mM NaCl, 142 mM KCl, 1 mM MgCl₂, 2 mM EGTA, 5.6 mM glucose and 25 mM HEPES, pH 7.2), equally distributed in two Eppendorf tubes and resuspended in previously warmed DMEM with 5% FCS and ± 2% EtOH. Parasite suspension was incubated at 37 °C for 30 minutes, followed by centrifugation at 1000 g, 4 °C, for 5 minutes. Supernatants (with constitutively or induced secreted micronemal proteins) were collected and centrifuged at 2000 g, 4 °C, for an additional 5 minutes to remove residual parasite debris. Pellets were washed once in PBS. Extracellular secreted antigens (ESA) and pellets were separated in a 12 % SDS-polyacrylamide gels and transferred to a nitrocellulose membrane. Membranes were blocked in 3% BSA/0.02% Tween in PBS (PBST) for 30 min. Western-blots were processed using a combination of primary antibodies for 60 min, washed and incubated with secondary antibodies an additional 60 min. Labelled membranes were detected using GE Healthcare Amersham ECL Western-Blotting Detection Reagent and visualized on a BioRad ChemiDoc TM MP Imaging system or directly visualized on a Li-Cor Odyssey infrared imager ODY 1060 v2.1.12, and the images processed with Image Studio v4.0.21.

Mouse infection
For each strain (wild type, Δdpy19 and Δdpy19comp), 5 CD1 mice (female, 6 weeks, Charles River Laboratories) were infected with 50 parasites by intraperitoneal injection (day 1). The health of the mice was monitored daily until they presented severe symptoms of acute toxoplasmosis (bristled hair and complete prostration with incapacity to drink or eat) and were sacrificed on that day. Since they showed no symptoms, the 5 mice infected with Δdpy19 parasites were challenged with 10⁵ wild type parasites at day 21.

Ethics statement
The animal experiments were conducted with the authorization number 1026/3604/2, GE30/l3 according to the guidelines and regulations issues by the Swiss Federal Veterinary Office. No human samples were used in these experiments.

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Conflict of interest
The authors declare that they have no conflicts of interest with the contents of this article.

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**Figure legends**

**Figure 1** – Deletion of *dpy19* abolishes C-mannosylation and leads to impaired cell invasion, parasite egress, motility and attachment. (A) Strategy for the targeted replacement of *Toxoplasma gondii* *dpy19* by the dihydrofolate reductase-thymidylate synthase (DHFR-TS) selection cassette mediated by homologous recombination. PCRs performed for validation of clones and the size of the expected product are indicated. (B) Validation of a ∆*dpy19* clone. Genomic DNA from the parental or ∆*dpy19* strain was used for PCR using the primer pairs AFA113/2017 (PCR1); 2018/AFA65 (PCR2); AFA01/AFA02 (PCR3) and AFA113/AFA65 (PCR4). (C) Western blotting of ∆*dpy19* and ∆*dpy19*comp total extracts labelled with an anti-Myc antibody confirms insertion and expression of the *dpy19* gene in the complemented mutant. (D) *Toxoplasma gondii* DPY19 localizes with the transiently expressed acetyl-CoA transporter AT1-Ty to the endoplasmic reticulum. Scale bars: 2 µm.

**Figure 2** – Absence of DPY19 leads to reduced MIC2 cellular level but does not impact on MIC2-M2AP complex formation, localization, secretion and proteolytic processing. (A) Total extract of wild type, ∆*dpy19* and ∆*dpy19*comp parasites were analyzed by Western blotting using an anti-MIC2 antibody (upper panel) and an anti-tubulin antibody (lower panel) to normalize loading in all lanes. MIC2 levels were normalized to tubulin and the average of three biological replicates is shown. One-way Anova with Tukey’s multiple comparison test * p<0.05; ns: non-significant (B) Co-immunoprecipitation of MIC2 and M2AP. M2AP was immunoprecipitated from wild type and ∆*dpy19* tachyzoites lysates with rabbit anti-TgM2AP. Immunoprecipitated proteins were then analyzed by Western blot labelled with mouse anti-TgMIC2 (upper panel) or rabbit anti-M2AP (lower panel). M2AP migrates as a proprotein (p) and mature protein (m). (C) Localization of the MIC2-M2AP complex in wild type and ∆*dpy19* tachyzoites. Scale bar: 2 µm. (D) Secretion and proteolytic processing of the micronemal proteins MIC2, AMA1 and MIC6. Wild type, ∆*dpy19* and ∆*dpy19*comp parasites were incubated in absence (-) or presence (+) of ethanol to induce microneme secretion. The resulting pellet and excretory-secretory antigens (ESA) were then analyzed by Western blot. Catalase (Cat.) was used as cytosolic control and dense granule 1 (GRA1) as control for constitutive secretion.

**Figure 3** – *T. gondii* ∆*dpy19* is strongly attenuated for virulence and confers protective immunity. Mice infected with 50 wild type or ∆*dpy19*comp parasites were sacrificed after 8 to 9 days due to severe symptoms, while all mice infected with 50 ∆*dpy19* parasites showed no symptoms. 21 days after the first parasite injection, the surviving mice were challenged with 100,000 wild type parasites and did not develop any sign of disease indicating that ∆*dpy19* confers protective immunity.
Figure 1
Figure 2
Figure 3
C-Mannosylation of *Toxoplasma gondii* proteins promotes attachment to host cells and parasite virulence

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