Myosin-dependent cell-cell communication controls synchronicity of division in acute and chronic stages of Toxoplasma gondii

Karine Frénal, Damien Jacot, Pierre-Mehdi Hammoudi, Arnault Graindorge, Bohumil Maco & Dominique Soldati-Favre

The obligate intracellular parasite Toxoplasma gondii possesses a repertoire of 11 myosins. Three class XIV motors participate in motility, invasion and egress, whereas the class XXII myosin F is implicated in organelle positioning and inheritance of the apicoplast. Here we provide evidence that TgUNC acts as a chaperone dedicated to the folding, assembly and function of all Toxoplasma myosins. The conditional ablation of TgUNC recapitulates the phenome of the known myosins and uncovers two functions in parasite basal complex constriction and synchronized division within the parasitophorous vacuole. We identify myosin J and centrin 2 as essential for the constriction. We demonstrate the existence of an intravacuolar cell–cell communication ensuring synchronized division, a process dependent on myosin I. This connectivity contributes to the delayed death phenotype resulting from loss of the apicoplast. Cell–cell communication is lost in activated macrophages and during bradyzoite differentiation resulting in asynchronized, slow division in the cysts.
Toxoplasma gondii, the causative agent of toxoplasmosis, belongs to the phylum of Apicomplexa that includes a number of human pathogens such as Plasmodium spp. responsible for malaria. Members of this phylum are obligate intracellular parasites that actively invade and egress from their target host cells using a substrate-dependent locomotion called gliding motility. The glideosome refers to the actomyosin system that powers forward movement of these parasites. It is located between the inner membrane complex (IMC) and the plasma membrane and composed of the class XIVα MyoA and gliding-associated proteins (GAPs)1. MyoA is conserved throughout the phylum2 and its depletion in T. gondii and P. berghei critically impacts motility, invasion and egress3–5. Conserved in the coccidian subgroup of Apicomplexa, the class XIVα TgMyoH initiates motility at the apical tip of the parasite likely by translocating adhesin-receptor complexes from the apex to the beginning of the IMC where TgMyoA takes the relay along the pellicle6–8. The class XIVb TgMyoC also restricted to the coccidians partially compensates for the deleterious but not lethal ablation of TgMyoA by its re-localization from the basal polar ring to the pellicle4,8,9. The class XXII TgMyoF is conserved across the phylum and indispensable for the correct positioning of the two centrosomes during cell division and the segregation of the apicoplast, a non-photosynthetic plastid-like organelle10. TgMyoF is additionally involved in the directed transport of secretory organelles called dense granules to the plasma membrane11 and positioning of the rhoptries to the parasite tip12. In parasites depleted in TgMyoF, organelles accumulate in enlarged residual bodies (RB)10, a structure derived from the mother cell postulated to contribute to the intravacuolar organization in rosette and as disposal of the remnants of the mother cell following division13. Parasites depleted in TgMyoF exhibit a ‘delayed death phenotype’ (DDP)10. This phenomenon described in T. gondii and Plasmodium spp., refers to perturbations of apicoplast functions by either pharmacological compounds or molecular genetic manipulation that result in loss of the organelle and in parasite death only on entry into the next lytic cycle14–16. T. gondii tachyzoite division is characterized by the synchronous formation and geometric expansion of two daughter cells within a mature mother cell; a process referred to endodyogeny17. Tachyzoites within a given vacuole tend to divide in a perfectly synchronized fashion, controlled by an unknown mechanism18.

Among the Apicomplexa, T. gondii possesses the largest repertoire of genes coding for myosin heavy chains with 7 of the 11 motors being uncharacterized to date. TgUNC is a myosin-specific co-chaperone of the UCS (UNC-45/CRO1/She4p) family19, which is critical for the heterologous production of soluble and functional TgMyoA20. UCS proteins interact with myosins of classes I, II, V and contribute to their stability21 raising the question about the importance of TgUNC in the folding of the 11 T. gondii myosins belonging to five distinct classes2. Like in other eukaryotes except fungi, TgUNC presents three tetratrico-peptide repeats (TPR) at its N-terminus followed by armadillo repeats that constitute the central and the UCS domains19,22. The UCS domain binds the myosin head domain while the TPR domain is able to interact with the general chaperone HSP90 or HSP70 (refs 23,24). The function of TgUNC of producing soluble and functional TgMyoA in insect cells was not dependent on the presence of the TPR motifs20 as similarly reported in Caenorhabditis elegans25.

In the present study, we demonstrate that conditional depletion of TgUNC results in the destabilization of the 11 myosins and complete block of parasite propagation. In addition to the severe defect in motility, invasion, egress and alteration in apicoplast inheritance, TgUNC-depleted parasites fail to constrict their basal pole and divide asynchronously within the parasitophorous vacuole (PV). To identify the myosins responsible for these two uncovered functions, each uncharacterized motor was functionally dissected. TgMyoJ, located at the posterior pole, is involved in the constriction of the basal complex and its deletion in the cyst-forming type II strain leads to a complete loss of virulence in the mouse model of infection. TgMyoI, located in an intercellular network as part of the RB, participates in the intravacuolar synchronicity of parasite division. Critically, TgMyoJ ensures cell–cell communication between intravacuolar tachyzoites, ensuring the synchronicity of division and shedding light on the mechanism of DDP. During stage conversion into bradyzoites and cyst formation, the connection between parasites is gradually lost and absent in cysts isolated from infected mice that replicate asynchronously.

Results

TgUNC ensures the assembly of all T. gondii myosins. The co-chaperone TgUNC was first conditionally knocked down via U1 snRNP-mediated gene silencing with the concomitant epitope tagging of the gene at the endogenous locus26 (Supplementary Fig. 1a). UNC-3Ty migrated at the expected size of 130 kDa (Fig. 1a and Supplementary Fig. 1f), was cytosolic (Fig. 1b) and almost fully extractable in presence of PBS (Supplementary Fig. 1b). The transient transfection of a vector expressing the Cre recombinase27 led to TgUNC depletion and loss of excised parasites after a few passages preventing the isolation of clones (Supplementary Fig. 1a). Next, CRISPR/Cas9 genome editing strategy was used to favour the replacement of the endogenous promoter with a tetracycline-repressive promoter (Supplementary Fig. 1c). The Myc-UNC-iKD clone was tightly downregulated with no TgUNC detectable by western blot (WB) analysis following 24 h of parasite cultivation in the presence of anhydrotetracycline (ATc) (Fig. 1c and Supplementary Fig. 11). ATc-treated parasites were severely affected in one or several steps of the lytic cycle since no lysis plaques were formed after 7 days (Fig. 1d). The defect was fully rescued by expressing either a second copy of TgUNC (UNC-Ty) or a truncated version lacking the 3 TPR motifs (UNCATPR-Ty), stably integrated under ATc selection (Supplementary Fig. 1d). Both UNC-Ty and UNCATPR-Ty localized to the cytosol (Supplementary Fig. 1e) and the inducible MycUNC was still regulated in complemented parasites (Supplementary Fig. 1f).

To determine their fate, all myosin heavy chains, except TgMyoA and TgMyoD, were C-terminally tagged at their endogenous locus in the MycUNC-iKD strain. TgMyoA was detected with specific α-TgMyoA antibodies, whereas for TgMyoD, antibodies detecting its specific myosin light chain TgMLC2 were used since TgMLC2 is known to readily disappear in the absence of TgMyoD28. On TgUNC depletion, all myosins were destabilized based on WB analysis, yet exhibiting different kinetics (Fig. 1e and Supplementary Fig. 11). In case of TgMyoD, a rapid disappearance of TgMLC2 was observed with almost undetectable level already 24 h after ATc treatment. TgMyoA, TgMyoE and TgMyoK were undetectable at 48 h after ATc treatment, while TgMyoC, TgMyoF, TgMyoG, TgMyoH, TgMyoI, TgMyoJ and TgMyoL levels were reduced but still detectable at this time point. Complementation of MycUNC-iKD with either UNC-Ty or UNCATPR-Ty restored the assembly of TgMyoA and presumably all the other myosins (Supplementary Fig. 1g).

Taken together, TgUNC is a cytosolic myosin-specific chaperone essential for the tachyzoite survival. The N-terminal TPR domain of TgUNC is dispensable for the ubiquitous role of TgUNC in the folding and stability of all the parasite myosins.
**Figure 1 | TgUNC is a myosin chaperone essential for tachyzoite survival.** (a) WB performed on total extract of extracellular tachyzoites, wild type (ΔKUB80) or expressing the endogenously tagged TgUNC (UNC-3Ty). Actin (ACT) was used as loading control. (b) UNC-3Ty was detected in the cytosol of intracellular tachyzoites co-stained with the peripheral marker GAP45. Scale bar, 2 μm. (c) The regulation of the Tet-inducible cell line of TgUNC (MycUNC-iKD) was assessed by WB on total extract of extracellular tachyzoites. ACT was used as loading control. (d) TgUNC depletion has a severe impact on parasite survival as shown by plaque assay treated for 7 days ± ATc. (e) All classes of myosin heavy chains are destabilized on TgUNC depletion. All the myosin motors, except TgMyoA and TgMyoD, have been endogenously tagged with 3xTy in the MycUNC-iKD strain and their expression level was followed by WB on total extracts of extracellular tachyzoites treated or not with ATc for 24 or 48 h. Expression of TgMyoD was followed using α-MLC2 antibodies. GAP40 or ACT were used as loading controls. MycUNC-iKD was tightly regulated in all samples. Stars indicate myosin subproducts. Uncropped gels are presented in Supplementary Fig. 11.

NATURE COMMUNICATIONS | DOI: 10.1038/ncomms15710 | www.nature.com/naturecommunications
TgUNC depletion sums up the phenotype of the known myosins. No defect in intracellular growth was observed 48 h after MycUNC depletion (Fig. 2a). In contrast and as anticipated from the rapid destabilization of TgMyoH, TgMyoA and TgMyoC, a severe block was observed in a gliding assay on poly-L-lysine-coated coverslips (Fig. 2b), as well as in invasion (Fig. 2c) and in egress (Fig. 2d) with less than 10% of entry and less than 5% of ruptured infected cells, respectively. Importantly, the PV membrane (PVM) was clearly ruptured attesting of the release of the perforin TgPLP1 and the lack of impact on microneme exocytosis (Supplementary Fig. 2a). In addition, TgMyoC, which is normally found exclusively at the basal polar ring, re-localized along the pellicle on TgUNC depletion (Supplementary Fig. 2b). This is in concordance with the differential kinetics of TgMyoA and TgMyoC destabilization (Fig. 1e) and the re-localization of TgMyoC while TgMyoA is deleted. The myosin light chain TgMLC1, which is shared by TgMyoA, TgMyoC and TgMyoH, was only partially downregulated at 48 h (Fig. 1e).

The apical structure composed of spirally arranged fibres made of tubulin and termed conoid was previously reported to protrude in motile parasites in a Ca\(^{2+}\)- and actin-dependent manner. Extracellular parasites depleted in MycUNC or treated with the actin depolymerizing agent cytochalasin D (CytD) still protruded their conoid on Ca\(^{2+}\)-ionophore treatment, arguing against the involvement of an actomyosin system in this process (Supplementary Fig. 2c).

TgUNC-depleted parasites showed no obvious division defect or alteration in biogenesis and positioning of micronemes, rhoptries, dense granules or mitochondrion (Supplementary Fig. 2d). However, these parasites failed to adopt the typical arrangement in rosettes and were clearly disorganized within their PV (Supplementary Fig. 2d), a phenomenon previously observed on TgMyoF depletion. A defect in apicoplast inheritance was visible by indirect immunofluorescence assay (IFA) using antibodies against the luminal plastid chaperone TgCpn60 (ref. 32) (Fig. 2e) with 30% of the vacuoles containing parasites lacking the apicoplast (Fig. 2f). In a less pronounced manner compared to TgMyoF depletion, parasites depleted in TgUNC also divided asymmetrically with the two daughter cells growing in opposite direction (up and down) instead of growing in the same orientation towards the apical end of the mother cell (Supplementary Fig. 2e). The modest phenotype is likely explained by the inefficient destabilization of TgMyoF even 48 h after TgUNC depletion (Fig. 1e). Collectively, TgUNC depletion recapitulates the phenotype of the known myosins.

**Basal constriction and synchronized division require TgUNC.** Strikingly, the localization of TgMyoC in TgUNC-depleted parasites revealed an enlarged basal polar ring of mature parasites despite the absence of a cytokinesis defect (Fig. 2g). Using MyoC as marker, the diameter of the basal pole was measured and shown to increase by 1.8-fold in parasites treated with ATc for 48 h (Supplementary Fig. 2f). The earliest marker of the basal complex, TgMORN1, appears just after the duplication of the centrosomes as a ring-like structure capping the basal end of the developing daughter cells, which then contracts and ultimately caps the basal pole of the mature parasites. TgMORN1 was endogenously GFP-tagged at its N-terminus in MycUNC-iKD parasites and shown as a dot at the centrocone, weakly at the apical end and strongly at the basal complex in non-dividing parasites (Fig. 2h). On TgUNC depletion, GFP-MORN1 localization was unchanged, however its basal staining delineated a larger ring that failed to contract. The EF-hand-containing protein centrin 2 (TgCEN2) is also known to be associated to the basal complex and was C-terminally YFP tagged at the endogenous locus in MycUNC-iKD parasites (Fig. 2i). TgCEN2 was visible at the apical end and annuli, and at the centrosome, however, the signal at the basal cup was absent in the absence of TgUNC. The morphological defect at the basal pole was examined by transmission electron microscopy (TEM). The basal complex appears as an electron-dense zone at the posterior ends of the IMC and such a zone was visible in the absence of TgUNC; however, the gap between the basal ends of the IMC was considerably larger compared to wild-type parasites confirming the impairment in constriction (Fig. 2j).

A second prominent defect on TgUNC depletion was revealed by IFA performed with antibodies against TgISP1 and TgIMC1, two proteins localized to the apical cap and the IMC of mature and daughter parasites, respectively. Remarkably, the high synchronicity of parasite division inside a given vacuole that is typically observed in wild-type tachyzoites was lost in MycUNC-iKD treated 48 h with ATc (Fig. 2k). The α-ISP1 and α-IMC1 were used to score progression of daughter cell formation and a loss in synchronicity was reported in at least 30% of TgUNC-depleted parasites (Fig. 2l).

Taken together, TgUNC-depleted parasites uncovered two new myosin-associated processes, the basal complex constriction and the synchronized division of intravascular parasites.

**TgMyo1 and TgMyoJ localize to the basal end of tachyzoites.** The localization of uncharacterized myosins was determined by IFA following C-terminal insertion of 3T4-epitope tags at the endogenous loci (Fig. 3a). TgMyoE was located to the conoid of daughter and mature parasites, whereas the unclassified TgMyoL was found to the conoid and cytosol. TgMyoG was weakly detectable at the periphery of the parasites. TgMyoK fluctuates along the cell cycle with a clear staining at the centrocones at the time when they duplicate as shown by its co-localization with TgMORN1. TgMyoL was found mainly in the RB, positioned predominantly at the centre of the rosettes. TgMyoJ was restrictively localized to the basal pole of developing daughters and mature parasites. The ring-like staining of TgMyoJ delineates the posterior end of the growing IMC that eventually constricts into a dot corresponding to the basal cup as shown by its co-localization with TgCEN2 (Fig. 3a).

Individual myosin knockouts were generated in the ΔKU80 parasites by interrupting the genes within the N-terminal head domain (Supplementary Fig. 3a,b). TgMyoE, G, J, K and L genes were individually disrupted without noticeable impact on parasite fitness, as monitored by plaque assay compared to their corresponding tagged version (Fig. 3b). Only MyoJ-KO parasites exhibited smaller plaques, indicative of a loss in fitness as confirmed by competition assay (Fig. 3c). Taken together, the presence of TgMyoJ at the basal pole and TgMyoL in an intercellular network suggests roles in basal constriction and parasites connection, respectively. Although it is not possible to formally exclude functional redundancies or adaptation mechanisms, the myosins characterized here are dispensable with only TgMyoJ necessary for optimal fitness.

**TgMyoJ is implicated in the basal complex constriction.** The positioning of the organelles in the myosin knockout mutants was not altered (Supplementary Fig. 4a); however, both MyoJ-KO and MyoL-KO parasites failed to develop into organized rosettes. In contrast to TgMyoF depletion, this disorganization did not correlate with an altered position of daughter cells growing in the up-and-down orientation during division (Supplementary Fig. 4b). IFA performed with α-IMC1 antibodies revealed a considerably enlarged posterior pole only in MyoJ-KO parasites (Fig. 4a and Supplementary Fig. 4a). Moreover, TgCEN2 was undetectable at the basal cup of MyoJ-KO and TgMyoC staining...
**Figure 2 | Impact of TgUNC depletion on tachyzoites.** (a) TgUNC depletion has no impact on intracellular growth. The number of parasites per vacuole was determined at 24 h after 48 h ± ATc. (b) TgUNC-depleted parasites are not able to glide as shown by the ionophore-induced gliding assay performed after 48 h ± ATc. Scale bar, 5 μm. (c) The invasion capacity of ΔKU80 and MycUNC-iKD strains was evaluated using a two-colour IFA performed after 48 h ± ATc. Parasites were allowed to invade for 30 min before fixing. (d) Ionophore-induced egress assay of ΔKU80 and MycUNC-iKD strains performed by treating the parasites with DMSO or A23187 for 7 min after 56 h ± ATc. (e) The presence of the apicoplast was assessed in MycUNC-iKD parasites using α-Cpn60 antibodies after 48 h ± ATc. Scale bar, 2 μm. (f) A slight defect in apicoplast inheritance was monitored in TgUNC-depleted parasites. (g–i) The posterior pole (arrowheads) of intracellular tachyzoites was examined using the endogenously tagged MyoC (g), MORN1 (h) or centrin2 (CEN2) (i) in the MycUNC-iKD strain treated ± ATc (48 h). Scale bar, 2 μm. (j) Electron micrographs of the basal pole of MycUNC-iKD parasites ± ATc (48 h). The arrowheads point to the basal ends of the IMC. N: nucleus. Scale bar, 1 μm. (k–l) The synchronicity of division was followed using α-ISPl and α-IMCl antibodies that stain the apical cap and the rest of the IMC, respectively (k) and quantified in ΔKU80 and MycUNC-iKD strains at 24 h after 48 h ± ATc (l). For a,c,d,f and l, the results are represented as mean ± s.d. from three independent experiments. Their significance was assessed using a parametric paired t-test and the two-tailed P-values are written on the graphs when significant.
appeared as enlarged as observed in the absence of TgUNC (Fig. 4a and Supplementary Fig. 2f). TEM confirmed the defect in constriction in the absence of TgMyoJ (Fig. 4b). Furthermore, the F-actin-binding protein coronin (TgCOR), previously shown to accumulate to the posterior pole of extracellular parasites on Ca^{2+}-ionophore treatment \(^3\) still re-localized at the basal pole in MyoJ-KO, indicating that TgMyoJ is not responsible for TgCOR re-localization in motile parasites (Supplementary Fig. 4c). TgMyoI was 3Ty-epitope tagged at the endogenous locus in MyoJ-KO and shown to accumulate at the enlarged posterior pole of the parasites but not detectable anymore in the RB (Fig. 4c).

**Figure 3 | Localization and essentiality of myosin heavy chains in tachyzoites.** (a) Subcellular localization of the myosin heavy chains endogenously tagged with 3xTy. The peripheral marker α-IMC1 and the cytoplasmic marker α-ACT were also used to visualize the parasites. In addition, TgCEN2 and TgMORN1 were endogenously tagged with YFP and Myc, respectively, in the MyoJ-3Ty and the MyoK-3Ty background. Scale bar, 2 μm. Dashed lines represent the parasite periphery. (b) Plaque assays performed over 7 days on the 3Ty-tagged (3Ty) and knockout (KO) strains of myosins. No defect of fitness was monitored in the KO strains comparatively to the corresponding tagged strains except for MyoJ-KO as confirmed by competition assay using GFP-expressing parasites as an internal control (c). The results are presented as mean ± s.d. from three independent experiments.

\(^{3}\) U80
Supplementary Fig. 3c) did not lead to an enhanced defect in parasite fitness (Supplementary Fig. 4d).

The perfect co-localization of TgCEN2 and TgMyoJ at the basal cup suggested that these two proteins might act together in basal constriction. Tet-repressive parasite line of TgCEN2 was generated using the same strategy as for MycUNC-iKD except that no N-terminal tag was introduced (Supplementary Fig. 3d). CEN2-iKD was subsequently C-terminally tagged with YFP to follow its downregulation on addition of ATc. After 48 h of treatment, CEN2-YFP-iKD was not detectable by WB (Fig. 4d and Supplementary Fig. 11) and only slightly detectable as weak dots at the centrosome (Fig. 4e). Depletion of TgCEN2 caused an...
enlarged posterior ring containing TgMyoJ without apparent defect in cytokinesis (Fig. 4f). As for MyoJ-KO, the same reduced constriction was measured using the endogenously tagged TgMyoC (Supplementary Fig. 2f). To assess the contribution of actin in this process, intracellular parasites expressing MyoJ-3Tyr and CEN2-YFP were treated for 4 h with 1 μM of CytD. The diameter of the ring staining of TgMyoJ increased by twofold in treated parasites compared to the untreated ones (Fig. 4g). Moreover, the posterior staining of TgCEN2 was no longer detectable, either lost or diluted along the enlarged basal cup.

These findings establish that TgMyoJ and TgCEN2 act in concert to constrict the basal complex at the end of division in an actin-dependent manner.

Impact of TgMyoJ and TgMyoI on residual body formation. To assess the formation of RB in absence of a proper marker of this structure, we deleted the Ca^{2+}-dependent protein kinase TgCDPK2, known to lead to an imbalance between starch synthesis and degradation and a dramatic accumulation of amylopectin granules in RB that can be visualized by periodic acid–Schiff (PAS) staining. TgCDPK2 was disrupted in MyoI-KO, MyoJ-KO and in ΔKU80 control strain without additional defect on the fitness of the parasites (Fig. 5a). In ΔKU80, amylopectin accumulated mainly in the RB at the centre of the rosettes as detected by PAS staining by differential interference contrast (DIC) (Fig. 5b), whereas it accumulated at the posterior but mainly inside the parasites in MyoI-KO/CDPK2-KO and MyoJ-KO/CDPK2-KO (Fig. 5b,c). TEM sections taken on intravacuolar parasites revealed that MyoI-KO/CDPK2-KO and MyoJ-KO/CDPK2-KO do actually form RB in which amylopectin accumulates but the parasites appear not to be connected to the RB in contrast to CDPK2-KO parasites (Fig. 5c).

Cell-cell communication ensures synchronized division. The loss of intravacuolar synchronized division observed in TgUNC-depleted parasites (Fig. 2l) was recapitulated in MyoI-KO and MyoJ-KO mutants based on detection of TgIMC1 and TgISP1 by IFA (Fig. 6a). The phenomenon becomes more pronounced as the number of division increases (Fig. 6b). In wild-type parasites, the loss of synchronicity was observed in large vacuoles (32 parasites/vacuole) when the rosette organization is compromised due to space constraint (Supplementary Fig. 5a). We reasoned that a connection must exist between tachyzoites within a vacuole, allowing soluble molecules such as cyclins or other cell cycle regulators to diffuse between parasites to tightly control the presence of the tubular mitochondrion within the connection passing through the basal complex whose diameter measures around 322 nm (Fig. 7c,d). Strikingly, the 3D reconstruction revealed the presence of the tubular mitochondrion within the connection between parasites/vacuole (Fig. 7b). TEM serial sections were used to perform 3D reconstruction of the vacuole (Fig. 7c,d). The raised the possibility that diffusion or exchanges could occur between parasites through the mitochondrion. To test it, the matrix superoxide dismutase 2 fused to GFP (Nt-SOD2-GFP) was used in the FRAP experiments; however, no recovery of fluorescence was observed in the >10 vacuoles tested (Supplementary Movie 8).

Given the key participation of TgMyoJ and TgMyoI in the formation and/or the maintenance of the connection between parasites, the importance of actin dynamics was assessed. Parasites treated for 2 h with 1 μM of CytD were still connected and partial loss of connection was observed when parasites were treated for 8–10 h with a fluorescence recovery indicating that only a subset of parasites remained connected within a given vacuole (Supplementary Fig. 5c and Supplementary Movie 9).

Collectively, parasite connectivity allows diffusion of soluble molecules between intravacuolar parasites and hence ensures synchronized division. This cell–cell communication is dependent on TgIMC1 and TgISP1. Only a long-term treatment with CytD that encompasses a complete division cycle impacts on connectivity suggesting the involvement of stable F-actin.
Cell–cell communication and delayed death phenotype.

The continuity between parasites allows the diffusion of soluble proteins and plausibly a large range of metabolites and even small vesicles. In this context, we examined the phenomenon of DDP, originally described in *T. gondii* tachyzoites, treated with clindamycin, ciprofloxacin or chloramphenicol. DDP is associated with the loss of apicoplast inheritance, which can be triggered by expression of dominant-negative mutants such as ACP-GFP-mROP1 (ref. 16) or DD-MyoF-tail. We reasoned that these mutants survived during the first lytic cycle due to the connection between parasites enabling the diffusion of one or more apicoplast-derived metabolites that can fuel all the parasites of the vacuole even if only few of them retained a functional apicoplast. To test this hypothesis, *TgMyoI* and *TgMyoJ* were individually deleted in parasites expressing stably DD-MyoF-tail on addition of shield (Shld-1) (Supplementary Fig. 6). The impact of DD-MyoF-tail expression on the various strains was assessed over 72 h and GFP-expressing parasites were mixed as an internal control (Fig. 8a). All the parasites grew like the GFP parasites in the absence of Shld-1. In contrast, the deleterious effect of DD-MyoF-tail expression was observed in the second lytic cycle in wild-type parasites but was already prominent in the first lytic cycle in DD-MyoF-tail/MyoI-KO and DD-MyoF-tail/MyoJ-KO parasites. Collectively, cell–cell communication confers resistance to the parasites lacking the apicoplast during the first lytic cycle and hence participates in the DDP (Fig. 8b).

Cell–cell communication is absent in mature tissue cysts.

The existence of an intravacuolar connection between parasites is not only a hallmark of the type I virulent strain RH since the observations were reproduced in the cyst-forming type II strain ME49 where both *TgMyoI* and *TgMyoJ* genes were disrupted (Supplementary Fig. 7a,b). Like in type I, type II ME49 MyoJ-KO tachyzoites formed slightly smaller lysis plaques after 10 days (Supplementary Fig. 7c) and exhibited a loss of fitness compared to wild-type and ME49 MyoI-KO parasites (Fig. 9a). While wild-type ME49 parasites exhibit a basal level of asynchronicity of division in about 15% of the vacuoles, this raised to more than 50% when *TgMyoI* or *TgMyoJ* were deleted and this asynchronicity increased with the number of divisions (Fig. 9b). Concordantly, FRAP experiments performed on GFP-expressing parasites confirmed that ME49 tachyzoites were connected in a *TgMyoI*- and *TgMyoJ*-dependent manner (Supplementary Fig. 7d–f and Supplementary Movie 10). During natural infection, tachyzoites rapidly encounter resident macrophages, dendritic cells and intraepithelial lymphocytes. Accordingly, we analysed cell–cell communication of both RH and ME49 tachyzoites in activated and non-activated bone marrow-derived macrophages (BMDMs). While RH parasites were largely connected (Fig. 9c, top panel and Supplementary Movie 11), ME49 parasites were only connected in non-activated BMDMs while communication was lost in activated BMDMs (Fig. 9c, bottom panel, Fig. 9d and...
Figure 6 | Intravacuolar TgMyoI-KO and TgMyoJ-KO parasites divide asynchronously and are not connected. (a) IFA showing the intravacuolar asynchronicity of division in MyoI- and MyoJ-KO stained with α-ISP1 and α-IMC1 24 h post infection. Scale bar, 2 μm. (b) Quantification of the synchronicity of intravacuolar division after 24 and 30 h of growth. Results are represented as mean ± s.d. from three independent experiments and the significance of the results was assessed using a parametric paired t-test. The two-tailed P-values are: 0.0219 (*) and 0.0175 (*) for MyoI-KO and MyoJ-KO, respectively, compared to ΔKU80 at 24 h and 0.0016 (**) and 0.0007 (***) at 30 h. (c) Transient transfection of SET8-HA shows that intravacuolar parasites are all in the same phase of their cell cycle in ΔKU80 in contrast to intravacuolar MyoI- and MyoJ-KO parasites. Scale bar, 2 μm. (d–h) Left panels: time-lapse imaging of FRAP experiments performed on type I tachyzoites ΔKU80 (d,e), Myo-KO (f) and MyoJ-KO (g) transiently transfected with GFP or on type I tachyzoites ΔKU80 transiently transfected with SET8-GFP (h). The bleached areas are delineated in red. Right panels: quantification of the intensity of GFP fluorescence recorded in the areas numbered on the left panels. Scale bars, 2 μm (d–g) and 5 μm (h). (i) Time-lapse imaging of a representative FRAP experiment performed on intracellular type I tachyzoites (RH-YFP) collected by intraperitoneal (IP) lavage 3 days p.i. (scale bar, 2 μm) and quantification of parasites connection in 10 vacuoles tested.
Supplementary Movie 11). The presence of a cyst wall marker, the *Dolichos biflorus* agglutinin (DBA) lectin was assessed under this experimental condition. While vacuoles formed by RH parasites are known not to be able to undergo stage conversion, ME49 vacuoles presented a basal level of DBA lectin staining both in activated and non-activated infected BMDMs (Fig. 9e). These observations suggest that the connection between parasites is dynamic and rapidly lost in parasite exposed to stress.

In vitro differentiated bradyzoites were reported to be asynchronus42 suggesting that bradyzoites are not connected. To investigate this further, ME49 tachyzoites were cultivated *in vitro* in conditions triggering differentiation into bradyzoites and maintained for several days to allow cyst formation as previously described43. The efficiency of stage conversion was assessed by IFA using α-P21 antibodies44 and the DBA lectin (Supplementary Fig. 8), and the proper formation of the cyst wall was verified by TEM (Fig. 10a). In bradyzoites differentiated *in vitro* during 14 days, we always observed asynchronicity of division with single parasite dividing within the cyst (Fig. 10b). FRAP experiments showed that in early cysts (from day 1 to day 6), parasites are connected two by two,
whereas after day 6, bradyzoites became progressively disconnected (Fig. 10c, Supplementary Fig. 9 and Supplementary Movies 12 and 13). In contrast, ME49 MyoI-KO and ME49 MyoJ-KO bradyzoites switched in vitro never appeared connected. These in vitro observations were confirmed on mature cysts collected from the brain of mice chronically infected with ME49-expressing GFP. Relevantly, the cyst-forming bradyzoite stage of T. gondii was reported to be less quiescent than previously assumed with isolated or patches of parasites often seen to divide asynchronously within cysts in vivo.\(^\text{45}\) The FRAP experiments performed on purified cysts showed no connection between bradyzoites even two by two (Fig. 10d and Supplementary Movie 14). The importance of TgMyoI and TgMyoJ as virulence factors in vivo was assessed by intraperitoneal infection of mice. Infection with either 500 or 1,000 tachyzoites of the type II ME49 wild-type or ME49 Myo-KO strains led to the death of the animals with the same time frame attesting that Myo-KO parasites are not impaired in virulence (Fig. 10e). In contrast, all the mice infected with type II ME49 Myo-J-KO survived infection without any apparent symptoms and fewer tissue cysts were detected from the brain of these animals compared to the control group and ME49 MyoI-KO parasites (Fig. 10f). The loss of virulence might result from a decreased mechanical resistance of Myo-J-KO compared to wild type or Myo-KO as observed when these parasites were subjected to a hypo-osmotic shock (Supplementary Fig. 10). Yet, the survival is apparently not dose-dependent since mice also survived infection with 150,000 parasites of the ME49 Myo-J-KO strain. Taken together, cell–cell communication is maintained in fast-replicating tachyzoites of both the type I and type II strains but is rapidly lost in the slow-growing bradyzoites.

Figure 8 | The intravacuolar connection contributes to the delayed death phenotype. (a) The survival of the DD-MyoF-tail, DD-MyoF-tail/MyoI-KO and DD-MyoF-tail/Myo-J-KO parasites was assessed at 24, 48 and 72 h p.i. in absence or presence of Shield-1 (Shld-1) using GFP-expressing parasites as an internal control. For comparison, the ratios of GFP control parasites at 24 h have been normalized to 20%. The results are expressed as mean ± s.d. (n = 3). (b) Model of parasites’ growth during the first lytic cycle depending on the presence or absence of TgMyoI and TgMyoJ and the defect (+ Shld-1) or not (− Shld-1) in apicoplast segregation. The diffusion of metabolites from the apicoplast (red dot) is represented in red. The different shades of red reflect the dilution of metabolites occurring on division when the apicoplast is not properly segregated.
Figure 9 | The connectivity between type II parasites shows higher susceptibility to stress conditions than type I. (a) Competition assay performed over six passages with type II ME49 wild type (WT), MyoI-KO and MyoJ-KO using GFP-expressing parasites as an internal control. As in type I, type II MyoJ-KO parasites exhibit a fitness defect. The results are expressed as mean ± s.d. (n = 3). (b) Quantification of the synchronicity of intravacuolar division 30 or 42 h p.i. Results are presented as mean ± s.d. (n = 3) and their significance was assessed using a parametric paired t-test. The two-tailed P-values are: 0.0120 (*) and 0.0025 (**) for MyoI-KO and MyoJ-KO, respectively, compared to WT at 30 h and 0.0057 (**)  and 0.0028 (**) at 42 h. (c) Time-lapse imaging of representative FRAP experiment performed activated BMDMs, 24 h after infection by type I (RH) tachyzoites (top panel) or 48 h after infection by type II (ME49) tachyzoites (bottom panel), all stably expressing GFP. Scale bar, 2 μm. (d) Quantification of parasites connection in n different vacuoles recorded from two biological replicates: n = 15 (RH, first column), n = 36 (ME49, second column), n = 26 (ME49, third column). Results are presented as mean ± s.d. and their significance was assessed using a parametric paired t-test. The two-tailed P-values are: 0.0224 (left *) and 0.0172 (right *). (e) Quantification of tachyzoite to bradyzoite conversion assessed by DBA lectin staining of RH-GFP (24 h p.i.) or ME49 (48 h p.i.) infected BMDMs, in 100 vacuoles from two biological replicates. Results are presented as mean ± s.d. and their significance was assessed using a parametric paired t-test. The two-tailed P-values are: 0.0168 (left *) and 0.0150 (right *).

Discussion

*T. gondii* possesses the largest repertoire of myosin motors among the members of Apicomplexa. TgUNC belongs to the UCS family of myosin chaperones and is critical for the assembly of all members of the five classes of *Toxoplasma* myosin heavy chains. TgUNC depletion recapitulates the phenotype of myosins implicated in motility, invasion and egress. These crucial steps of the lytic cycle rely on the concerted action of TgMyoH, TgMyoA and TgMyoC and no additional motor participating in motility was uncovered here, although functional redundancy or compensatory mechanisms cannot be excluded. The defect in apicoplast inheritance previously assigned to TgMyoF was only modestly reproduced in the absence of TgUNC, probably due to the incomplete disappearance of TgMyoF. Importantly, no myosins appear to play a critical role in parasite division as was previously but only indirectly deduced from CytD treatment. The RB varies in size depending on parasite fitness and especially on its ability to undergo proper division with accurate positioning of the organelles. Typically, enlarged RBs were previously observed in parasite treated with drugs disrupting actin polymerization, on perturbation of TgMyoF function or when illegitimate amylopectin granules are formed on disruption of TgCDPK2 (ref. 37). In the absence of TgMyo or TgMyoJ, parasites do not form a RB except when forced to do so by deletion of TgCDPK2. This result is in concordance with the fact that the dramatic accumulation of mislocalized organelles in the RB monitored in absence of TgMyoF was not recapitulated in parasites depleted in TgUNC. TgMyoI, a class XXIV myosin, is the first reported to affect the localization of TgMyoJ. Since TgCEN2 is a small EF-hand-containing protein, it could conceivably act as myosin light chain for TgMyoJ and modulate its function to generate the contractile force or alternatively act as cargo and be brought to the site of action by TgMyoJ. Despite this spectacular morphological phenotype, the apparently ‘truncated’ parasites survived albeit with a loss of fitness.

Following invasion and sealing of the PVM, *T. gondii* tachyzoites divide asexually by a process called endodyogeny and implying that the two daughter cells are built inside the mother parasite. At the end of the division, the daughter cells separate at their posterior pole but appear to remain connected via the RB that constitutes a vacuolar network which maintains the parasites spatially organized in rosettes within the PV during the subsequent rounds of division. The RB varies in size depending on parasite fitness and especially on its ability to undergo proper division with accurate positioning of the organelles. Typically, enlarged RBs were previously observed in parasite treated with drugs disrupting actin polymerization, on perturbation of TgMyoF function or when illegitimate amylopectin granules are formed on disruption of TgCDPK2 (ref. 37). In the absence of TgMyo or TgMyoJ, parasites do not form a RB except when forced to do so by deletion of TgCDPK2. This result is in concordance with the fact that the dramatic accumulation of mislocalized organelles in the RB monitored in absence of TgMyoF was not recapitulated in parasites depleted in TgUNC. TgMyoI, a class XXIV myosin, is the first reported marker of this vacuolar compartment and is mislocalized in MyoI-KO parasites. This plausibly explains the dual phenotype observed in absence of TgMyoJ and points towards TgMyoJ being the motor responsible for the formation/maintenance of the connection. The RB contributes along with the orientation of the

Discussion

*T. gondii* possesses the largest repertoire of myosin motors among the members of the five classes of *Toxoplasma* myosin heavy chains. TgUNC depletion recapitulates the phenotype of myosins implicated in motility, invasion and egress. These crucial steps of the lytic cycle rely on the concerted action of TgMyoH, TgMyoA and TgMyoC and no additional motor participating in motility was uncovered here, although functional redundancy or compensatory mechanisms cannot be excluded. The defect in apicoplast inheritance previously assigned to TgMyoF was only modestly reproduced in the absence of TgUNC, probably due to the incomplete disappearance of TgMyoF. Importantly, no myosins appear to play a critical role in parasite division as was previously but only indirectly deduced from CytD treatment. The RB varies in size depending on parasite fitness and especially on its ability to undergo proper division with accurate positioning of the organelles. Typically, enlarged RBs were previously observed in parasite treated with drugs disrupting actin polymerization, on perturbation of TgMyoF function or when illegitimate amylopectin granules are formed on disruption of TgCDPK2 (ref. 37). In the absence of TgMyo or TgMyoJ, parasites do not form a RB except when forced to do so by deletion of TgCDPK2. This result is in concordance with the fact that the dramatic accumulation of mislocalized organelles in the RB monitored in absence of TgMyoF was not recapitulated in parasites depleted in TgUNC. TgMyoI, a class XXIV myosin, is the first reported marker of this vacuolar compartment and is mislocalized in MyoI-KO parasites. This plausibly explains the dual phenotype observed in absence of TgMyoJ and points towards TgMyoJ being the motor responsible for the formation/maintenance of the connection. The RB contributes along with the orientation of the
daughter cells to the arrangement in rosette of the tachyzoites within the PV. The TEM-3D reconstruction of a vacuole allowed the visualization of the structure responsible for the connection and confirmed that intravacuolar parasites indeed share the same cytosol. This connection and concomitantly the synchronized division are lost in absence of TgMyoI or TgMyoJ. The FRAP experiments clearly demonstrate the existence of a cell–cell communication between intravacuolar tachyzoites that allows the diffusion of soluble cytosolic GFP as well as the nuclear cell cycle regulated H4K20 methylase TgSET8. The diffusion of GFP and TgSET8 strongly suggests that all sorts of soluble metabolites can diffuse between parasites, including metabolites produced by the apicoplast and postulated to participate in the DDP phenomenon. Concordantly, parasites lacking the apicoplast as consequence of impairment of TgMyoF function are more severely affected during the first lytic cycle if they lack either TgMyoI or TgMyoJ. Similarly, in Plasmodium, the DDP could simply be explained by the shared cytoplasm allowing the diffusion of metabolites until the parasites segmented at the very last step of schizogony. The presence at high frequency of the tubular mitochondrion in the figure is not relevant for the discussion of the text.
connection as observed by TEM serial sections suggests possible exchanges between parasites through this organelle. However, this does not occur at the level of matrix protein as shown with SOD2-GFP. Interestingly, in mammalian cells, mitochondria have been observed within nanotubular cell-to-cell connections termed membrane nanotubes enabling the exchange of material, energy, but also signals between cells48 and in analogy, most of the membrane nanotubes contain actin filaments.

Importantly, the connectivity in *T. gondii* is observed between intravacuolar tachyzoites of both type I (RH) and type II (ME49) strains and is not maintained during development of the cyst-forming bradyzoites. Specifically, type II strain is sensitive to stress stimuli and prone to lose connection when exposed to activated macrophages. In this context, disconnected parasites might be preparing for bradyzoite differentiation. The FRAP experiments performed on advanced in vitro differentiated parasites as well as on in vivo tissue cyst show that bradyzoites are clearly not connected. This finding correlates with the recent observation of low level of synchronized division of bradyzoites within cysts formed from the central nervous system of mice45. Interestingly, during the initial phase of bradyzoite division, the parasites remained connected two by two, which explains why TgMyoL is expressed in bradyzoite stage. It is plausible that the link established by TgMyoL at the end of the endodyogeny process is actively broken to slow down the growth of the cyst and participate in the process of latency during chronic infection.

The lack of connection observed in type II MyoL-KO tachyzoites had no impact on virulence compared to wild type in the mice model. In contrast, type II ME49 Myo-KO are unable to kill the mice even at relatively high doses. Concordantly very few cysts were detectable in the brain of mice infected with ME49 Myo-KO parasites although they appeared morphologically normal. The reduced fitness possibly attributed to loss of mechanical resistance observed in the competition assays and also reported in the recent CRISPR/Cas9 screen of the *T. gondii* genome49 might explain the phenomenon although further investigation is needed to unravel the nature of this lack of virulence.

The implication of a myosin in the connectivity between two cells raises a number of challenging questions about the nature of the exchange. The enlarged basal pole and asynchronous division can be also observed in TgACT1-iKO parasites50. In this study, chromodobies that specifically bind F-actin, detected filaments in the RB and emanating from the basal pole of parasites confirming the involvement of the actomyosin system in parasite connectivity and communication. Finally, a key step of the lytic cycle is the egress from infected cells with the concomitant switch of parasites to the motility mode. Individualization of the parasites appears to be a prerequisite for survival and spreading and thus the RB must collapse with proper sealing of the parasites at the time of egress. In consequence, signalling cascade leading to microneme secretion, motility and parasites sealing must operate in a tight, timely concerted fashion, for example by responding simultaneously to signalling molecules such as Ca2+ and/or cGMP.

**Methods**

Preparation of *T. gondii* genomic DNA and RNA. Genomic DNA (gDNA) has been prepared from tachyzoites (RH and ME49 strains obtained from aidsreagent.org and ATCC, numbers are 50174 and 50611) using the Wizard SV genomic DNA purification system (Promega).

Cloning of DNA constructs. All amplifications for cloning were performed with the LA Taq (Takara) or Q5 (New England Biolabs) polymerases and the primers used are listed in Supplementary Table 1. All amplifications for screening were performed with the GoTaq DNA polymerase (Promega) and the primers are listed in Supplementary Table 2.

To introduce epitope tag to myosins, their endogenous loci were modified with ‘knock-in’ vectors. The gDNA fragment of the C-terminal part of TgMyoE (TGME49_239560), TgMyoG (TGME49_314780), TgMyoL (TGME49_230980), TgMyoM (TGME49_257470) and TgMyoK (TGME49_206415) was amplified by PCR using the primers listed in Supplementary Table 1, digested with the restriction enzymes listed in Supplementary Table 1 and cloned into KpnI or MfeI and NsiI sites of the pU8BMIC13-3Ty-HX (ref. 51). For PCR amplification, the plasmids have been linearized in the middle of the cloned gDNA fragment. For knock-in insertion of these vectors into the UNC-iKO strain, the HXGPRK cassette has been exchanged with a DHFR-TS cassette subcloned into the two SacII sites. For TgMyoL (TGME49_290120), a gDNA fragment has been amplified using the primers TgMyoM-35489-TgMyoM-35494 digested with Apal and SflI and cloned into the Apal and NsiI sites of the pU8BMIC13-3Ty-HX (ref. 51). For PCR amplification, the plasmids have been linearized in the middle of the cloned gDNA fragment. For knock-in insertion of these vectors into the UNC-iKO strain, the HXGPRK cassette has been exchanged with a DHFR-TS cassette subcloned into the two SacII sites. For TgMyoL (TGME49_290120), a gDNA fragment has been amplified using the primers TgMyoM-35489-TgMyoM-35494 digested with Apal and SflI and cloned into the Apal and NsiI sites of the pU8BMIC13-3Ty-HX (ref. 51). For PCR amplification, the plasmids have been linearized in the middle of the cloned gDNA fragment. For knock-in insertion of these vectors into the UNC-iKO strain, the HXGPRK cassette has been exchanged with a DHFR-TS cassette subcloned into the two SacII sites.

To generate TgMyoL-KO and TgMyoK-O in the type I ME9AHAH strain, the CRISPR-Cas9 system was used to disrupt the genes by insertion of a DHFR-TS selection cassette into the coding sequence of the head domain. To do so, a PCR product was generated using the KOD DNA polymerase (Novagen, Merk) with the vector p2834-DHFR (ref. 54) as template and the primers TgMyoL-6022/TgMyoL-6023 or TgMyoL-6024/TgMyoL-6025 that also carry 30 bp homology with the respective myosin locus. To do the insertion of the gDNA fragment specific to this myosin, a gDNA fragment that has been generated for each myosin using the Q5 site-directed mutagenesis kit (New England Biolabs) and the vector pSAG1-CAS9-GFP-U6: sgUPRT as template57. The UPRT-targeting gRNA was replaced by a TgMyoL-specific gRNA using the transfection pair TgMyoL-6022/TgMyoL-6023 and TgMyoL-6024/GRNA-4883, respectively. To generate TgMyoL-KO and TgMyoK-KO in the DD-MyoF-tail strain, the same specific sgRNA vectors were used with the CRISPR-Cas9 system to disrupt the genes. Parasites expressing the Cas9-YFP were sorted by flow cytometry (FACS) and cloned into 96-well plates using a MoFlo Astrios (Beckman Coulter). The clones were then analysed by sequencing.

To generate the UNC-3Ty-LoxP-2′UTR-LoxP-U1 vector, a gDNA fragment corresponding to the C terminus of TgUNC (TGME49_249480) was amplified by PCR using the primers TgUNC-3565/TgUNC-3567 and cloned into the KpnI and NsiI sites of the p0152-3Ty-LoxP-2′UTR-Srag1-HXGPRK-Loxp-U1 plasmid56 previously modified to introduce a unique KpnI site and a sequence coding for a 3′-tag of the fragment.

To generate tet-repressive knockdown of TgUNC, a PCR fragment encodes the TATi trans-activator, the HXGPRK cassette and the TetO7S promoter was generated using the KOD DNA polymerase (Novagen, Merk) with the vector 5′MyoF-TATi-HX-tetO7S1MycNtMyoF10 as template and the primers TgUNC-3565/TgUNC-3567 that also carry 30 bp homology with the 5′ end of TgUNC. To direct the insertion of the PCR product at the start of TgUNC, a specific sgRNA vector has been generated as described above using the primer pair TgUNC-3566/GRNA-4883.

To generate TgMORN1-KO in *T. gondii* strains, the coding sequences of TgMORN1 and TgUNCAT98 were amplified from gDNA using the primers TgMORN1-3561-3335/TgUNCAT98-3567 and cloned into the head domain thus creating a truncated and non-functional protein. A gDNA fragment of the head domain of TgMyoG, TgMyoL, TgMyoK and TgMyoL was amplified by PCR using the primers listed in Supplementary Table 1 and inserted into the KpnI and NsiI restriction sites of the pU8BMIC13-3Ty-HX (ref. 51).

To generate TgUNC expressing constructs for complementation experiments, the coding sequences of TgUNC and TgUNCAT98 were amplified from gDNA using the primers TgUNC-5690/TgUNC-5697 and TgUNC-5690/TgUNC-5707, respectively, and then digested with MfeI and NsiI for cloning into the EcoRI and NsiI sites of the pUT88MycGFPPPMamoAtiy-HX vector58. These plasmids were linearized with BglI before transfection.

To generate TgMORN1-KO in *T. gondii* strains, the coding sequences of TgMORN1 and TgUNCAT98 were amplified from gDNA using the primers TgMORN1-3561-3335/TgUNCAT98-3567 and cloned into the head domain thus creating a truncated and non-functional protein. A gDNA fragment of the head domain of TgMyoL was then endogenously C-terminally tagged with YFP using the pLIC-CEN2-YFP-CAT vector57 linearized with EcoRV.
Toxoplasma gondii
WB analysis
in triplicates and 5–10 vacuoles were bleached each time. performed with 100% of laser (wavelength 488) and another acquisition step of composed of an acquisition step of three images followed by two bleaches previously described 56. Confocal images were generated with a Zeiss LSM700 or paraformaldehyde (PFA) or 4% PFA/0.05% glutaraldehyde (PFA/GA) in PBS, 16
ARTICLE NATURE COMMUNICATIONS | DOI: 10.1038/ncomms15710
pT8-MycGFPPfMyoAtail-Ty56 was amplified using primers SET8-6989 and vector38 digested with the same enzymes.
m follow: polyclonal rabbit:
a
HXGPRT56, pTub-SOD2-Nterm-GFP-Myc (Nt-SOD2-GFP) 40 and GFP-HA-SET8
FRAP
Geneva. Stacks of sections were processed with ImageJ and projected using the LSM800 laser scanning confocal microscope using an apochromat/C2 63/1.4 oil
conversion from tachyzoite to bradyzoite was induced by replacing normal media with RPMI 1640 buffered with 50 mM HEPES to pH 8.2 and supplemented with 3% foetal bovine serum. Parasites were allowed to grow at 37°C in absence of CO2; for multiple days and alkaline media was changed daily.

Parasite transfection and selection of stable transformants. Parasite transfections were performed by electroporation as previously described38. RHAKU80 (ref. 53) and derivative strains have been transfected with 15–20 μg of the knock-in constructs. Either mycophenolic acid (MPA, 25 mg ml⁻¹) and xanthine (50 mg ml⁻¹) or pyrimethamine (1 mg ml⁻¹) was used to select the resistant parasites carrying the HXGPRT or the DHFR cassette, respectively. For the CRISPR-Cas9 strategy, 15 μg of the gRNA-specific CRISPR/CAS9 vector was transfected into RHAKU80 together with the product of two PCR reactions and selected according to the selection cassette used. UNC-1KD strain was complemented with 60 μg of linearized pTub8-UNC-Ty-HX or pTub8-UNCATPR-Ty-HX under ATc selection. The hgxprt locus has been disrupted into the type II ME49 strain using the CRISPR-Cas9 system and a specific sgRNA generated as previously described with the primer pair gRNA-HX-5950 and gRNA-4883. Parasites expressing the Cas9 enzyme were sorted by flow cytometry (FACS) and the clones were analysed by sequencing (Supplemental Fig. 6a). In this background, TgMyoI was endogenously tagged using the pKI-Myl-5T-HX-GHPRT construct described above and in the same background as well as in the derived Myo-KO and Myo-KO, the pTub-GFP-HX-GHPRT construct39 was stably integrated after Neo linearization.

Antibodies. The antibodies used in this study were previously described as follows: polyclonal rabbit: α-MyoA and α-MLC1 (ref. 39), α-GAP40, α-IMC1 and α-GAP2 (ref. 9), α-CP60 (ref. 32), α-catalase (α-Go)2, α-HSP70 (ref. 40); monoclonal mouse, α-HSP70 (ref. 53) and derivative strains have been transfected with 15–20 μg of linearized pTub8-UNC-Ty-HX or pTub8-UNCATPR-Ty-HX under ATc selection. The hgxprt locus has been disrupted into the type II ME49 strain using the CRISPR-Cas9 system and a specific sgRNA generated as previously described with the primer pair gRNA-HX-5950 and gRNA-4883. Parasites expressing the Cas9 enzyme were sorted by flow cytometry (FACS) and the clones were analysed by sequencing (Supplemental Fig. 6a). In this background, TgMyoI was endogenously tagged using the pKI-Myl-5T-HX-GHPRT construct described above and in the same background as well as in the derived Myo-KO and Myo-KO, the pTub-GFP-HX-GHPRT construct39 was stably integrated after Neo linearization.

IFAs. Parasite-infected HFF cells seeded on coverslips were fixed with 4% paraformaldehyde (PFA) or 4% PFA/0.05% glutaraldehyde (PFA/GA) in PBS, depended of the antigen to be labelled. Parasites were then processed as previously described60. Confocal images were generated with a Zeiss LSM700 or LSM800 laser scanning confocal microscope using an apochromat × 63/1.4 oil objective at the Bioimaging core facility of the Faculty of Medicine, University of Geneva. Stacks of sections were processed with ImageJ and projected using the maximum projection tool.

FRAP. For FRAP experiments performed on tachyzoites, the pTub-GFP-HX-GHPRT66, pTub-SOD2-Nterm-GFP-Myc (Nt-SOD2-GFP)60 and pTub8-SET8 plasmids were transiently transfected into the parasites and the experiments were performed 24 h later for the RH strains or 48 h later for the ME49 strains. For the experiments performed on bradyzoites, ME49 strains stably expressing GFP were used and bradyzoite stage conversion was induced for 1–15 days. The experiments were performed on a Nikon A1R microscope (Ti Eclipse) controlled in temperature and CO2. Acquisitions and processing were done with the softwares NS-elements advanced research (Nikon) and ImageJ. The sequence used for the experiments was composed of an acquisition step of three images followed by two bleaches performed with 100% laser (wavelength 488) and another acquisition step of 3–15 min with images collected every 5 or 10 s. All experiments were done at least in triplicates and 5–10 vacuoles were bleached each time. All experiments were performed at the Bioimaging core facility of the Faculty of Medicine, University of Geneva.

WB analysis. Parasites were lysed in RIPA buffer (150 mM NaCl, 1% Triton X-100, 0.5% deoxycholate, 0.1% SDS, 50 mM Tris pH 7.5), incubated on ice for 15 min and then centrifuged for 30 min at 14,000 r.p.m. at 4°C. The supernatant was then collected and mixed with SDS–PAGE loading buffer under reducing conditions. Separated proteins were transferred to nitrocellulose membranes and probed with appropriate antibodies in 5% non-fat milk power in PBS-0.05% Tween20. Bound secondary peroxidase-conjugated antibodies were visualized using the ECL system.

Plaque assay. Confluent HFF cells were infected with freshly egressed parasites and treated ± ATc if necessary. After 7–10 days, the cells were fixed with PFA/GA and stained with a crystal violet solution (Sigma).

Intracellular growth and synchronicity of division assays. Freshly egressed parasites were inoculated on confluent HFFs and allowed to grow for 24 h before fixation with PFA/GA. For MycUNC-iKD parasites, a pre-treatment ± ATc was performed 24 h before egress and continued for 24 h before fixation with PFA/GA. For intracellular growth assay, IFAs were performed using ± GAP45. The number of parasites per vacuole was determined by counting the parasites in 100 vacuoles in duplicate for three independent experiments. The data are presented as mean ± s.d.

To determine the synchronicity of cell division within the vacuoles, double-labeling IFAs were performed using ± IS1 and ± IMC1 antibodies. The development of the daughter cells was evaluated in 100 vacuoles in duplicate for three independent experiments. For type I RHAKU80, Myo-KO and Myo-KO, fixation has been done 24 and 30 h post invasion, for type II strains, fixation has been done 30 and 42 h post invasion. The results are presented as mean ± s.d.

Competition assay. Type I and type II WT, Myo-KO and Myo-KO parasites were mixed with GFP-expressing parasites at a ratio of about 80/20. This ratio was then determined over 10 passages by IFA using ± GAP45. At each passage, 100 vacuoles were counted in duplicate from three biological replicates. The ratios have been normalized to 80% at t0. The data are presented as mean ± s.d.

Red/green invasion assay. MycUNC-iKD parasites were pre-treated for 48 h ± ATc before performing the assay as previously described59. The number of intracellular and extracellular parasites was determined by counting 100 parasites in duplicate for three independent experiments. The results are presented as mean ± s.d.

Induced egress assay. Freshly egressed MycUNC-iKD parasites were inoculated on HFF cells and allowed to grow for 30 h ± ATc before adding either the calcium ionophore A23187 (3 μM) or DMSO for 7 min as previously described61. Double-labeling IFA was performed using ± GRA3 and ± GAP45 antibodies. The average number of egressed vacuoles was determined by counting 100 vacuoles in duplicate for each condition and for three independent experiments. The results are presented as mean ± s.d.

Induced gliding assay. MycUNC-iKD parasites were grown for 48 h ± ATc. Freshly egressed parasites were settled on poly-l-lysine-coated coverslips in DMEM by centrifugation 1 min at 1,000 r.p.m. and then incubated for 15 min in an HEPES/calcium-saline solution with calcium ionophore A23187 (3 μM) before fixation with PFA/GA. ± SAG1 antibody was used without permeabilization to visualize the trails and the parasites. Three independent experiments have been performed.

Apicoplast segregation assay. Freshly egressed RHAKU80 and MycUNC-iKD parasites, pre-treated ± ATc for 24 h, have been inoculated on new HFFs and allowed to grow for 24 h ± ATc before fixation with PFA/GA. Double-labeling IFA was performed using ± CP60 and ±-actin antibodies. The number of vacuoles with a correct segregation of the apicoplast was determined by counting the parasites and the apicoplasts in 100 vacuoles in duplicate for three biological replicates. The data are presented as mean ± s.d.

Delayed death phenotype. Freshly egressed parasites of the DD-Myo-tail, DD-Myo-tail/Myo-KO and DD-Myo-tail/Myo-KO strains were mixed with GFP-expressing parasites at a ratio of 80/20. These extracellular parasites were then treated for 3 ± Shield-1 before allowing them to invade new HFFs under the same treatment. The ratio of non-GFP/GFP parasites was then assayed intracellularly at 24 and 72 h and extracellularly at 48 h. Hundred vacuoles or parasites in duplicate from four independent experiments were counted. The ratios non-GFP/GFP have been normalized to 80/20 at 24 h. The results are presented as mean ± s.d.

TEM. Freshly egressed parasites (KO-Myo and KO-Myo) were inoculated on confluent HFFs and allowed to grow for 24 h while UNC-iKD parasites were first pre-treated ± ATc for 24 h before egress and then inoculated on new confluent HFFs and allowed to grow for 24 h ± ATc. Infected host cells were washed with 0.1 M PBS pH 7.4, then fixed with 2.5% glutaraldehyde in 0.1 M PBS pH 7.4, scraped and pelleted. Samples were then treated as previously described66. Thin sections were analysed using a Tecnai 20 electron microscope (FEI Company) at the ‘Pôle
Faculté de Microscope Ultrastructurale (PFMU) of the Faculty of Medicine of Geneva.

**Serial sections TEM.** HFF cells infected with RHAKU° parasites were grown in HFF monolayer on round (12 mm) glass coverslips for 24 h. Cells were fixed with 2.5% GA/2% PFA (Electron Microscopy Sciences) in 0.1 M phosphate buffer (PB) at pH 7.4 for 1 h at room temperature. Cells were extensively washed with 0.1 M cacodylate buffer, pH 7.4 and post fixed with 1% osmium tetroxide (Electron Microscopy Sciences) and 1.5% potassium ferrocyanide in 0.1 cacodylate buffer, pH 7.4 for 40 min followed by 1% osmium tetroxide (Electron Microscopy Sciences) alone in 0.1 M cacodylate buffer pH 7.4 for additional 40 min. Cells were then washed twice for 5 min in double distilled water and en block stained with aqueous 1% uranyl acetate (Electron Microscopy Sciences) for 1 h. After 5 min wash in double-distilled water, cells were dehydrated in graded ethanol series (2 × 50%, 70%, 90%, 95% and 2 × absolute ethanol) for 3 min each wash. Cells were then infiltrated with mixed series of Durcupan resin (Electron Microscopy Sciences) diluted with ethanol at 1:2, 1:1 and 2:1 for 30 min each, and twice with pure Durcupan for 30 min each. Cells were infiltrated with fresh Durcupan resin for additional 2 h and the coverslips were placed with grown cells and twice with pure Durcupan for 30 min each. Cells were then infiltrated with graded series of Durcupan resin (Electron Microscopy Sciences) coated with Formvar support film. A glass slide coated with mould-separating agent and filled with fresh resin. This sandwich was homogenized in 1 ml PBS with 1% Tween and syringe passaged five times through a 16G needle to break up large clumps. Then, the homogenate was sequentially syringe passed through an 18G needle (5 times), a 20G needle (10 times) and a 23G needle (10 times). Tissue cysts number was estimated by counting five frames of 10 μl from each brain homogenate using the ×20 objective of an inverted microscope.

**Ethics statement.** All animal experiments were conducted with the authorization number 1026/3604/2, GE30/13 according to the guidelines and regulations issues by the Swiss Federal Veterinary Office. No human samples were used in these experiments.

**Data availability.** All relevant data are available from the authors on request.

**References**

1. Heintzelman, M. B. Gliding motility in apicomplexan parasites. Semin. Cell Dev. Biol. 46, 135–142 (2015).
2. Forst, B. J., Goedecke, M. C. & Soldati, D. New insights into myosin evolution and classification. Proc. Natl Acad. Sci. USA 103, 3681–3686 (2006).
3. Meissner, M., Schloter, D. & Soldati, D. Role of Toxoplasma gondii myosin A in powering parasite gliding and host cell invasion. Science 298, 837–840 (2002).
4. Andenmatten, N. et al. Conditional genome engineering in Toxoplasma gondii uncovers alternative invasion mechanisms. Nat. Methods 10, 125–127 (2013).
5. Sebastian, S. et al. A Plasmodium calcium-dependent protein kinase controls zygote development and transmission by translationally repressing mRNAs. Cell Host Microbe 12, 9–19 (2012).
6. Siden-Kiamos, I. et al. Stage-specific depletion of myosin A supports an essential role in motility of malarial oocinets. Cell. Microbiol. 13, 1996–2006 (2011).
7. Graindorge, A. et al. The conoid associated motor MyoH is indispensable for Toxoplasma gondii entry and exit from host cells. PLoS Pathog. 12, e1005388 (2016).
8. Engert, S. et al. The toxoplasma Acto-MyoA motor complex is important but not essential for gliding motility and host cell invasion. PLoS ONE 9, e91819 (2014).
9. Frenal, K., Marq, J. B., Jacot, D., Polonais, V. & Soldati-Favre, D. Plasticity of MyoC- and MyoA-glisomes: an example of functional compensation in Toxoplasma gondii invasion. PLoS Pathog. 10, e1004504 (2014).
10. Jacot, D., Daher, W. & Soldati-Favre, D. Toxoplasma gondii myosin F, an essential motor for centrosomes positioning and apicoplast inheritance. EMBO J. 32, 1702–1716 (2013).
11. Heaslip, A. T., Nelson, S. R. & Warshaw, D. M. Dense granule trafficking in Toxoplasma gondii requires a unique class 27 myosin and actin filaments. Mol. Biol. Cell 27, 2080–2089 (2016).
12. Mueller, C. et al. The Toxoplasma protein ARO mediates the apical positioning of rhoptry organelles, a prerequisite for host cell invasion. Cell Host Microbe 13, 299–301 (2013).
13. Muniz-Hernandez, S. et al. Contribution of the residual body in the spatial organization of Toxoplasma gondii tachyzoites within the parastrophorous vacuole. J. Biomed. Biotechnol. 2011, 473983 (2011).
14. Dahl, E. L. & Rosenthal, P. J. Multiple antibiotics exert delayed effects against Toxoplasma gondii. Antimicrob. Agents Chemother. 51, 3485–3490 (2007).
15. Ficher, M. E. & Roos, D. S. A plastid organelle as a drug target in apicomplexan parasites. Nature 390, 407–409 (1997).
16. He, C. Y. et al. A plastid segregation defect in the protozoan parasite Toxoplasma gondii. EMBO J. 20, 330–339 (2001).
17. Francia, M. E. & Striepen, B. Cell division in apicomplexan parasites. Nat. Rev. Microbiol. 12, 125–136 (2014).
18. Nishi, M., Hu, K., Murray, J. M. & Roos, D. S. Organellar dynamics during the cell cycle of Toxoplasma gondii. J. Cell Sci. 121, 1559–1568 (2008).
19. Barral, J. M., Bauer, C. C., Ortiz, I. & Epstein, H. F. Unc-45 mutations in Caenorhabditis elegans implicate a CROI/Shep-like domain in myosin assembly. J. Cell Biol. 143, 1215–1225 (1998).
20. Bookwalter, C. S., Kelsen, A., Leung, J. M., Ward, G. E. & Trybus, K. M. A Toxoplasma gondii class XIV myosin, expressed in Sf9 cells with a parasite co-chaperone, requires two light chains for fast motility. J. Biol. Chem. 289, 30832–30841 (2014).
21. Wesche, S., Arnold, M. & Jansen, R. P. The UCS domain protein Shep4 binds to myosin motor domains and is essential for class I and class V myosin function. Curr. Biol. 13, 715–724 (2003).
22. Ni, W. & Odunuga, O. O. UCS proteins: chaperones for myosin and co-chaperones for Hsp90. Subcell. Biochem. 78, 133–152 (2015).
23. Barral, J. M., Hateganu, A. H., Brinker, A., Hartl, F. U. & Epstein, H. F. Role of the myosin assembly protein UNC-45 as a molecular chaperone for myosin. Science 295, 669–671 (2002).
24. Hellerschied, M. & Clausen, T. Myosin chaperones. Curr. Opin. Struct. Biol. 25, 9–15 (2014).
53. Huynh, M. H. & Carruthers, V. B. Tagging of endogenous genes in a Toxoplasma gondii strain lacking Ku80. *Eukaryot. Cell* 8, 530–539 (2009).

54. Roos, D. S., Donald, R. G., Morrissette, N. S. & Moulton, A. L. Molecular tools for genetic dissection of the protozoan parasite Toxoplasma gondii. *Methods Cell Biol.* 45, 27–63 (1994).

55. Chen, B., Brown, K. M., Lee, T. D. & Sibley, L. D. Efficient gene disruption in diverse strains of *Toxoplasma gondii* using CRISPR/CAS9. *mbio* 5, e01114–e01114 (2014).

56. Hettmann, C. et al. A dibasic motif in the tail of a class XIV apicomplexan myosin is an essential determinant of plasma membrane localization. *Mol. Biol. Cell* 11, 1385–1400 (2000).

57. Lenti, G. et al. Identification and characterization of *Toxoplasma* SIP, a conserved apicomplexan cytoskeleton protein involved in maintaining the shape, motility and virulence of the parasite. *Cell Microbiol.* 17, 62–78 (2015).

58. Soldati, D. & Boothroyd, J. C. Transient transfection and expression in the obligate intracellular parasite *Toxoplasma gondii*. *Science* 260, 349–352 (1993).

59. Hern-Gotze, A. et al. *Toxoplasma gondii* myosin A and its light chain: a fast, single-headed, plus-end-directed motor. *EMBO J.* 21, 2149–2158 (2002).

60. Ding, M., Clayton, C. & Soldati, D. *Toxoplasma gondii* catalase are there peroxisomes in *toxo*? *J. Cell Sci.* 113, 2409–2419 (2000).

61. Beck, J. R. et al. A novel family of *Toxoplasma* IMC proteins displays a hierarchical organization and functions in coordinating parasite division. *PLoS Pathog.* 6, e1001094 (2010).

62. Bastin, P., Bagherzadeh, Z., Matthews, K. R. & Gull, K. A novel epitope tag system to study protein targeting and organelle biogenesis in *Trypanosoma brucei*. *Mol. Biochem. Parasitol.* 77, 235–239 (1996).

**Acknowledgements**

We gratefully acknowledge the assistance and support of Jean-Baptiste Marq for the documentation of Em pictures and Julian Salaman for his early contribution to MyoC characterization (CMU, Faculty of Medicine, University of Geneva). Dr Maryse Lebrun (University of Montpellier) is acknowledged for providing the LIC-Centren2-YFP-CAT vector. Dr Markus Meissner (University of Glasgow) for providing the U1 vector, Dr David Sibley (Washington University) for the CRISPR/Cas9 plasmid, Dr Mohamed-Ali Halimi for the SET8 vector (University of Grenoble) and Yalim Emre for reagents (M-CSF).

This research was supported by the Swiss National Foundation to D.S.-F. (FN310040-116722 and CRSII3_160702). D.S.-F. is an International Scholar of the Howard Hughes Medical Institute. K.F. received funding from the ‘Sir Jules Thorn Charitable Overseas Trust fund’, Schaan’s subsidy for young researchers. Results incorporated in study received funding from the European Research Council (ERC) under the European Union’s Horizon 2020 research and innovation programme under Grant agreement no. 695996.

**Author contributions**

K.F., D.J. and D.S.-F. designed experiments; K.F., D.J., A.G., P.-M.H. and D.S.-F. performed experiments; K.F., D.J., A.G., P.-M.H. and D.S.-F. analysed the data; K.F., D.J., D.S.-F. wrote the manuscript.

**Additional information**

Supplementary Information accompanies this paper at http://www.nature.com/naturecommunications

Competing interests: The authors declare no competing financial interests.

Reprints and permission information is available online at http://npg.nature.com/reprintsandpermissions

How to cite this article: Frenal, K. et al. Myosin-dependent cell-cell communication controls synchronicity of division in acute and chronic stages of *Toxoplasma gondii*. *Nat. Commun.* 8, 15710 doi: 10.1038/ncomms15710 (2017).

Publisher’s note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.