A circular zone of attachment to the extracellular matrix acts as a guidance system for *Toxoplasma gondii* 3D motility

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Abstract

Toxoplasma gondii is a protozoan parasite that infects 30-40% of the world's population. Infections are typically subclinical but can be severe and, in some cases, life threatening. Central to the virulence of *T. gondii* is an unusual form of substrate-dependent motility that enables the parasite to invade cells of its host and to disseminate throughout the body. A hetero-oligomeric complex of proteins that functions in motility has been characterized, but how these proteins work together to drive forward motion of the parasite remains controversial. A key piece of information needed to understand the underlying mechanism(s) is the directionality of the forces that a moving parasite exerts on the external environment. The linear motor model of motility, which has dominated the field for the past two decades, predicts continuous anterior-to-posterior force generation along the length of the parasite. We show here using three-dimensional traction force mapping that the predominant forces around a moving parasite are instead periodic and directed in towards the parasite at a fixed circular location within the extracellular matrix. These highly localized forces, which are generated by the parasite pulling on the matrix, create a visible constriction in the parasite's plasma membrane. We propose that this circular ring of inward-directed force corresponds to a circumferential attachment zone between the parasite and the matrix, through which the parasite passes as it moves forward. In parasites lacking the major surface adhesin, TgMIC2, neither the inward-directed forces nor the constriction of the parasite membrane are observed. TgMIC2-deficient parasites remain capable of moving at speeds similar to wildtype but the mutant parasites move in a less directed fashion, suggesting that TgMIC2-mediated circumferential adhesion of the parasite to the extracellular matrix serves as a guidance system to maximize forward displacement during parasite motility.

Introduction

Parasites of the phylum Apicomplexa cause pervasive human disease and are responsible for millions of deaths annually. *Toxoplasma gondii* is the most prevalent apicomplexan parasite, infecting 30-40% of the human population worldwide [1-3]. *T. gondii* infections are typically subclinical in immunocompetent individuals but may cause blindness [4, 5]. In immunocompromised individuals and the developing fetus, the disease can be life-threatening [3, 6, 7].

T. gondii is a single-cell protozoan, and acute disease is caused by the highly motile tachyzoite life-cycle stage. Like other apicomplexan parasites, the *T. gondii* tachyzoite uses a unique form of substrate-dependent motility to invade into and egress from cells of its host, to migrate across biological barriers, and to disseminate through the infected host. Motility is, therefore, central to the pathogenesis of toxoplasmosis [8-10]. The parasite can move at speeds of up to 3 μm/s without the cilia, flagella, or leading edge protrusions that drive the motility of other eukaryotic cells. Within a three-dimensional (3D) extracellular matrix, the parasite moves along a complex helical trajectory characterized by regular oscillations in velocity and changes in trajectory curvature and torsion [11].

Motility is driven, at least in part, by a complex of proteins known as the glideosome, which is anchored to the inner membrane complex at the parasite periphery (see Suppl. Fig. 1). In the "linear motor" model of motility, short actin filaments are bound through a bridging protein to the cytosolic tails of transmembrane adhesins, such as TgMIC2, and translocated towards the posterior end of the parasite via a class XIV myosin, TgMyoA [12-15]. If the extracellular domains of the transmembrane adhesins are attached to ligands on the substrate, the rearward translocation of actin results in forward movement of the parasite. Other apicomplexan parasites, including those that cause malaria (*Plasmodium spp.*) and cryptosporidiosis, express similar glideosome proteins, suggesting that the mechanism of motility is conserved [16].

Although abundant evidence supports the importance of TgMyoA and other components of the glideosome in motility [10, 14, 17-21], several recent observations have raised questions about whether these proteins generate force as described by the linear motor model [17, 22-26] and, if so, whether this is the only motility mechanism available to the parasite. For example: (a) parasites conditionally depleted of TgMyoA and other glideosome components show greatly reduced motility but are not completely immotile [17, 22, 25], and (b) the linear motor model predicts uniformly forward directed motion, but under some conditions parasites are seen to repeatedly switch the directionality of their movement [27-30].

Because parasite motility is essential for virulence, elucidation of the underlying mechanism(s) is of both fundamental cell biological interest and potential clinical relevance. One key piece of missing information needed for a full mechanistic understanding of motility is the directionality of the forces the

parasite exerts on the external environment as it moves along its 3D helical trajectory. The linear motor model predicts continuous anterior-to-posterior forces along the entire periphery of the moving parasite. We show here using 3D traction force mapping that the predominant forces exerted by the parasite are instead organized into a highly localized circumferential ring of inward-directed force at a fixed location within the matrix. These inward-directed forces create a visible constriction of the parasite's plasma membrane, reflecting a circular zone of tight attachment of the parasite to the matrix through which the parasite passes through as it progresses forward along its helical trajectory. In the absence of this circular attachment zone, the parasite is still able to move but this movement loses much of its directionality.

Results

Development of a quantitative 3D traction force mapping assay

As a first step towards visualizing the forces exerted by parasites moving in 3D, we used a bead displacement assay in the well-established Matrigel extracellular matrix model [11, 25, 31]. Fluorescent microspheres embedded within the Matrigel were pulled towards a moving parasite from nearby anterior, posterior, and lateral locations within the matrix, and these microspheres returned to their original positions after the parasite had passed (Figure 1A, Videos 1&2). These results demonstrate that the parasite does indeed exert a detectable inward force on the surrounding matrix. Unexpectedly, we also noted constrictions in the body of the fluorescent parasite as it moved (Figure 1A, Video 2). These constrictions were even more apparent by brightfield microscopy (Fig. 1B, Video 3). Each constriction formed at the apical end of the parasite and remained stationary relative to the matrix as the parasite moved through, reminiscent of the "moving junction" through which the parasite penetrates during host cell invasion (discussed further below).

To develop a more quantitative force mapping assay and explore the relationship between the constrictions and matrix deformation, we used Alexa-Fluor 647-conjugated fibrinogen [32] to generate a fluorescent 3D fibrin matrix (Suppl. Figure 2A), enabling the mapping of matrix displacements at all spatial points in our imaged volume rather than at the limited number of discrete positions offered by microspheres. Parasite motility in matrices generated using 2.25 mg/ml fibrinogen was indistinguishable from that observed in Matrigel (Suppl. Figure 2B, C). Visual comparison of successive image volumes confirmed that parasites moving through fluorescent fibrin also deform the matrix (Suppl. Figure 2D and Video 4) and undergo the striking constrictions seen in Matrigel.

To calculate and map the deformations of the fibrin matrix, we used the Fast Iterative Digital Volume Correlation (FIDVC) algorithm developed by Christian Franck's lab (https://github.com/FranckLab/FIDVC) to iteratively determine the 3D displacement fields between

consecutive volumetric image stacks in our time series [33]. Each image volume is divided into 16,807 subvolumes (49 x 49 x 7 subvolumes in *x*, *y*, *z*). The algorithm compares the voxel intensity pattern between corresponding subvolumes at two consecutive timepoints, calculating one 3D displacement vector per subvolume. The data can be displayed as a map for the entire imaging volume (*e.g.*, Video 5) or for any 2D plane (*x-y*, *x-z*, or *y-z*). Most of the 2D images below show the *x-y* displacements on one of the seven *z* subvolume levels.

To filter out background signal and identify displacements caused by the parasite, we used timepoints when no parasites were moving to calculate a displacement detection threshold for each individual dataset, eliminating concerns about run-to-run variation (see Methods and Suppl. Figure 3A). Using this approach, the limit of detection for 3D displacements in the system ranges from 0.042-0.046 µm; a similar 3D displacement detection threshold (0.043-0.046 µm) was observed in samples containing no parasites (Suppl. Figure 3B), validating the approach. When analyzing data in the *x-y* plane only, the background cutoff was calculated using the *x-y* displacement magnitudes (rather than the *x-y-z* displacement magnitudes); this lowered the displacement detection threshold to 0.028-0.031 µm.

In order to translate matrix displacements into the magnitude of forces generated by the parasite, we determined the viscoelasticity of the fibrin matrix using laser trapping (see Methods). The fibrin gel behaves as a predominantly elastic matrix (Suppl. Figure 4) with an elastic modulus of 15.6 pN/µm and a viscous modulus of 0.09 pN*s/µm. The minimal detectable force magnitude in this system is therefore 0.66-0.72 pN in 3D and 0.45-0.49 pN in the *x-y* plane.

Parasite constrictions are tightly linked to periodic bursts of matrix deformation and motility

With this system for 3D traction force mapping in hand, we analyzed the pattern and directionality of forces produced by wild-type parasites as they moved through the fibrin matrix. Plotting fibrin displacement as *x-y* maps showed that the matrix deforms in towards the parasite from all *x-y* directions at discrete, periodic time points along the trajectory, before relaxing back to its initial position (*e.g.*, Figures 2A-D and Videos 6, 7). Viewing the data as projections along each axis demonstrates that the matrix is simultaneously pulled in towards the parasite in all three dimensions (Suppl. Figure 5). In approximately 50% of cases, inward deformation occurred between two consecutive image volumes (1.6 seconds apart) and was followed in the next image volume by relaxation (*e.g.*, Figure 2A-C); in the remainder of cases the deformation was held through multiple time points (*e.g.*, Figure 2D) before relaxing (Suppl. Figure 6). During periods when the matrix was not being pulled inward or relaxing outward, no deformations of the matrix above background were detectable (Suppl. Figure 3A). This observations suggests either that the parasite only pulls on the matrix during some portions of its trajectory or that the parasite produces both

strong and weak deformations, and the weak deformations – which could be continuous – are below our level of detection.

When the displacement maps were overlaid onto the images of the moving parasites, it became immediately apparent that the timing of each large matrix deformation coincided with a parasite constriction and that the displacement vectors pointed primarily in towards the constriction (Figure 2C, D; additional examples are shown in Suppl. Figure 7). The fact that the displacements were directed inwards rather than away from the parasite indicates that the pinching of the parasite membrane results from pulling forces generated by the parasite rather than the parasite squeezing through a narrow pore within the matrix, which would push the matrix away. When a constriction persisted over multiple time points, the fibrin continued to deform into the constriction and/or to hold the deformation over those same time points as the parasite passed through (*e.g.*, Figure 2D).

The time it took a parasite to move completely through a constriction ranged from 1.6-56.2 seconds (average 6.8 ± 5.2 seconds, measured in brightfield) with the longer times due to a stall in forward progression of a constricting parasite. The time between one constriction finishing and another beginning was also variable (Figure 3A). In 46.3% of the cases, the end of one constriction was followed immediately by the beginning of another, while in 13.8% of the cases (n=188) a second constriction would start before the first constriction finished (Figure 3A, B and Video 8). Most importantly, all parasites that move at least one body length undergo a constriction and the total distance moved is directly proportional to the number of constrictions observed (Figure 3C), strongly suggesting that the parasite-generated forces that create the constriction play an important role in forward movement.

The much reduced width of the parasite during a constriction (2.56 ± 0.38 µm at its widest point before constriction *vs.* 1.39 ± 0.24 µm during constriction; Figure 3D) led us to question whether the constriction presents a physical obstacle to the forward motion of the nucleus (normal nuclear diameter measured by DNA staining = 2.00 ± 0.26 µm). In parasites labeled with both a fluorescent DNA stain and a fluorescently-labeled antibody against the major surface protein, TgSAG1 (to visualize the constriction), the normally round parasite nucleus did indeed become thinner and more elongate as it passed through the constriction and regained its round shape once it was through (Figure 3E). The ratio of the nuclear dimensions perpendicular and parallel to the long axis of the parasite was 0.93 ± 0.08 in a non-constricting parasite and 0.50 ± 0.06 during a constriction. We examined whether the periodic need to squeeze the nucleus through the constriction might be responsible for the periodic changes in speed observed along the parasite's trajectory [11], but tracking analysis of constricting parasites did not reveal any consistent relationship between the instantaneous speed of the parasite and position of the nucleus relative to the constriction.

Parasites that do not generate constrictions do not detectably deform the matrix

Since TgMyoA is thought to play a central role in force production during parasite motility (Suppl. Figure 1), we conducted similar force mapping experiments using a parasite line lacking TgMyoA [17, 18]. As previously reported [25], these parasites are much less motile in 3D than wildtype: only 18 of 769 TgMyoA knockout parasites examined (2.3%) moved one body length or more, compared to 99 of 278 wild-type parasites (35.6%; Suppl. Figure 8A). Of the 18 TgMyoA knockout parasites that moved at least one body length, none showed constrictions like those seen in wild-type parasites. The pattern of movement was also different from wild-type parasites; rather than moving along smooth helical trajectories, the TgMyoA knockout parasites moved in tightly twisting arcs or in a stair-like pattern consisting of right-angled turns associated with a sharp bend in the body of the parasite (Suppl. Figure 8B and Video 9). In stark contrast to the wild-type parasites, we also saw no evidence by traction force mapping of a ring of inward-directed forces produced by the TgMyoA knockouts. In fact, we did not detect any displacement of the matrix by these parasites (Suppl. Figures 3C and 8C-E), suggesting that any forces produced by the moving knockout parasites are below the limit of detection in our traction force assay.

We also tested whether the surface adhesin, TgMIC2, plays a role in forming the circular zone of attachment to the matrix and matrix deformation. The motility defect in parasites lacking TgMIC2 is less severe than the defect in parasites lacking TgMyoA, with 36 of 297 TgMIC2 knockout parasites (12.1%) capable of moving more than one body length (see also [31, 34]). However, these parasites often moved in an erratic, stop and start pattern, bending sharply and moving in tightly twisting arcs (Figure 4A and Video 10). Moreover, the TgMIC2 knockout parasites also failed to form constrictions and did not detectably deform the fibrin matrix (Fig. 4A, Suppl. Figures 3D & 9C-E) despite being able to move at maximum speeds similar to wild-type parasites [31]. Taken together, these data confirm that motility is significantly altered (but not completely eliminated) in parasites lacking TgMyoA or TgMIC2 and demonstrate that these proteins are required to produce the constriction and the constriction-associated inward forces on the matrix observed during the motility of wild-type parasites.

TgMIC2-deficient parasites move less directionally

The larger number of TgMIC2 knockout parasites capable of long runs of motility enabled us to analyze their phenotype in greater detail. The nuclei of the parasites lacking TgMIC2 did not change shape as they moved in 3D (Figure 4B and Suppl. Figure 9A), providing further evidence that these parasites do not undergo the constrictions seen in wild-type parasites. Given their apparent propensity for tight turns, we also quantified the ability of the TgMIC2 knockout parasites to move directionally, by comparing displacement distance to trajectory length. While parasites with and without TgMIC2 traveled

along trajectories of similar mean length (38.47 ± 9.3 μm vs. 42.22 ± 8.0 μm, respectively), parasites lacking TgMIC2 move approximately half as far from their starting point as wild-type parasites (Fig. 4C and Suppl. Figure 9B). These data suggest that the circumferential zone of attachment to the matrix, which requires TgMIC2, functions as a guidance system, converting meandering motility into more linear, directed forward progression.

Discussion

The linear motor model of apicomplexan motility predicts that a parasite moves by pulling continuously on the matrix in an anterior-to-posterior direction along the length of the parasite. It was, therefore, unexpected that the predominant forces revealed by our new 3D traction force mapping assay were instead periodic and highly localized, pointing inwards from all directions towards a specific circumferential location on the parasite surface. This circular band of inward-directed force forms at a fixed position within the matrix and creates a visible constriction at the parasite periphery. The constriction passes from the anterior to the posterior end of the parasite as the parasite moves forward. Typically, when the constriction reaches the parasite's posterior end a new constriction forms at the apical end and the cycle repeats. These results suggest that the moving parasite periodically assembles ring-shaped zones of tight attachment to the matrix, which deform the matrix and serve as stationary platforms that the parasite propels itself through, one body length at a time.

Parasite attachment to the substrate and force generation in 2D *vs.* 3D. Both malaria sporozoites and *T. gondii* tachyzoites are capable of moving in circles on 2D surfaces. The pioneering work of Munter, Frischknecht and colleagues combined traction force mapping with reflection interference contrast microscopy (RICM) during the 2D circular gliding of malaria sporozoites [35]. They concluded that the parasite adheres to the substrate at its apical and posterior ends; each of these adhesion sites produces stalling forces parallel to the long axis of the parasite, and disengagement of the adhesion sites drives forward motion. Traction forces perpendicular to the direction of movement were also detected at the center of the parasite, but these were interpreted as nonspecific and nonproductive. The apical and posterior adhesion sites were shown to move forward *with* the parasite, rather than remaining stationary with respect to the substrate.

T. gondii tachyzoites demonstrate a second form of productive motility on 2D surfaces called helical gliding: the banana-shaped parasite starts with its left side in contact with the substrate, moves forward one body length while rotating along its longitudinal axis until it lies on its right side, and then flips back to its left side to repeat the cycle [36]. This form of motility is the equivalent of the fully helical trajectories seen in 3D but constrained in 2D by contact of the curved parasite with the rigid substrate. In RICM studies of 2D helical gliding [19, 37] the tachyzoite was seen to first attach to the substrate at its

apical end. The zone of attachment then expanded along the length of the parasite and, as the parasite moved forward and its apical end twisted up off the cover slip, the zone of attachment shrunk until it was reduced to a spot at the posterior end. In contrast to *Plasmodium* sporozoites, the position of the tachyzoite adhesion site remained fixed relative to the substrate and the parasite slid over this attachment site as the parasite moved forward. 2D traction force mapping revealed forces parallel, but not perpendicular, to the longitudinal axis of the tachyzoite; these traction forces, together with spring-like forces generated by the parasite's microtubule cytoskeleton, were postulated to produce forward motion [37].

A consensus emerges when these previous studies of *T. gondii* 2D motility are combined with the 3D results presented here that the basic unit of motility consists of apical attachment to the substrate, followed by apical-to-posterior translocation of the adhesion site, which is fixed in its position relative to the substrate. When the adhesion site reaches the basal pole of the parasite, a new apical attachment site is formed, and the cycle begins again. The adhesion site in the 3D matrix is circular, and the pulling forces exerted by the parasite on the matrix manifest as a visible constriction in the parasite's body. In 2D, no deformation of the parasite has been reported at the adhesion site, likely reflecting differences in attachment to the substrate via a point/small patch on the parasite surface *vs.* circumferentially. In 2D, attachment of the apical end of the tachyzoite to the substrate was associated with a sharp bend or "kink" in the apical tip of the parasite [37]. These kinks were more apical than the whole body bends we observed in the TgMyoA and TgMIC2 knockout parasites, and they resolved rather than passing down the length of the parasite as the constriction does in 3D. We also saw no evidence in 3D for a persistent stall force at the rear of the parasite. The biggest difference between the 2D and 3D force mapping data is that, in 3D, all force vectors point towards one highly localized circumferential region on the surface of the parasite, and many of these force vectors contained a strong component perpendicular to the long axis of the parasite.

3D motility and invasion: variations on a theme. During the invasion of host cells by *T. gondii*, proteins secreted from the parasite's apical organelles assemble a ring-shaped zone of tight attachment between the membranes of the two cells. This "moving junction" does not in fact move: it is anchored to the host cytoskeleton and is hypothesized to provide a fixed platform against which the parasite exerts force [38-42]. Parasite surface adhesins, which are engaged with the junction via their extracellular domains, are thought to be translocated by TgMyoA in an anterior-to-posterior direction, pushing the parasite through the junction and into the host cell. The body of the parasite narrows dramatically as it passes through the moving junction, bearing a striking resemblance to the constrictions seen during 3D motility.

Two central players in moving junction formation are TgAMA1, an adhesin secreted onto the parasite surface from the micronemes, and TgRON2, which is secreted by the parasites into the host cell plasma membrane and serves the ligand to which TgAMA1 binds. Neither TgAMA1 [43, 44] nor TgRON2 (RVS and GEW, unpublished data) plays any detectable role in parasite motility. Instead, TgMIC2 – another microneme protein secreted onto the parasite surface – may play an analogous role during motility to that of TgAMA1 during invasion, attaching the parasite to ligands in the extracellular matrix rather than to TgRON2 in the host cell membrane.

As the parasite moves forward during both invasion and 3D motility, it simultaneously rotates around its long axis [11, 36, 45]. Our results provide a possible explanation for this rotational motion. In both cases, parasite receptors bind to their cognate ligands in the form of a circular ring of attachment that is fixed in position relative to the host cell (in the case of invasion) or the extracellular matrix (in the case of motility). It has long been proposed that the TgMyoA motor driving invasion and motility is arranged helically within the parasite, along the spiraling subcortical microtubules (*e.g.*, [37]). Alternatively, a recent modeling study suggests that the unique geometry of the parasite periphery can generate a self-organized helical pattern of anterior-to-posterior actin flow, without the need for the TgMyoA motor to be organized in a helical pattern (Hueschen *et al*, submitted). In either case, if the actin filaments flow helically and are connected to the tails of transmembrane adhesins engaged circumferentially with the host cell surface or extracellular matrix, the forward motion of the parasite into the cell or through the matrix will by definition be accompanied by rotational twist. Some portion of the perpendicular force that we observed directed towards the constriction may in fact reflect torque on the matrix produced by the parasite as it twists through this circular zone of attachment.

The parallels between invasion and 3D motility noted here raise the intriguing possibility that the characteristic constriction of the parasite seen at the moving junction during invasion does not result from the parasite being squeezed as it *pushes* itself through the small diameter opening into the host cell, as is commonly thought (*e.g.,* [46, 47]), but rather to the parasite attaching to and *pulling* on the host cell plasma membrane as it penetrates.

The linear motor model. The data presented here with wild-type parasites are at least partially consistent with the linear motor model of motility, in which: (a) actin polymerization is nucleated at the apical tip of the parasite by formin1 [19]; (b) TgMyoA translocates the actin filaments in an anterior-to-posterior direction along the length of the parasite; and (c) binding of the rearward flowing actin filaments to transmembrane adhesins [14] whose extracellular domains are attached to the substrate drives the forward motion of the parasite (Suppl. Figure 1). While anterior-to-posterior actin flux may occur along the entire length of the parasite periphery (Hueschen *et al*, submitted), we propose that force is generated, at least in part, by the rearward translocation of the subset of actin filaments that are coupled to adhesins

at the localized ring of attachment. We were unable to detect any anterior-to-posterior bias in the force vectors at the constriction, which would be expected if the parasite pulls against this zone of attachment in order to move forward. These forces may be below our limit of detection (~0.72 pN). Because the fibrin gel behaves primarily as an elastic matrix (Suppl. Figure 4), the relationship between acceleration and force is complex and we cannot calculate with any confidence how much total force / displacement would be required to achieve a given parasite velocity. Alternatively, compression of the fibrin immediately adjacent to the constriction might increase the local elastic modulus of the matrix, making it more difficult to see proximal displacements because of a local increase in the displacement detection threshold.

The remarkable regularity with which the parasite generates the circular zones of attachment on its surface, yielding on average one constriction per body length of motion, could be due to repetitive bursts of formin1-mediated actin polymerization at the parasite's apical tip [19] or to periodic release of adhesins such as TgMIC2 from the micronemes. Alternatively, the observation that constrictions occur back-to-back almost 50% of the time (Fig. 3A), may suggest that a new constriction forms at the apical tip before the previous constriction has ended, but this new constriction stalls there as the TgMyoA (or possibly TgMyoH [19]) to which it is connected pulls against the adhesive forces of the previous constriction. Future real-time analysis of actin polymerization and microneme secretion in motile parasites and higher sensitivity force mapping will be necessary to distinguish between these possibilities and further interrogate the periodic nature of ring assembly.

The lack of a motility-associated constriction in TgMyoA-deficient parasites could reflect an inability either to assemble ring of adhesins at the apical tip of these parasites or, more likely, to move through the incipient ring once the ring has formed. Alternatively, it could be that the ring-shaped structures that form during motility are contractile, and contractility requires TgMyoA. The inward deformation of the fibrin matrix towards the constriction reported here is consistent with a contractile ring, as is the physical constriction of the parasite plasma membrane. Further experiments will be required to distinguish between these possibilities.

If the coupling of actin flow to the circular zone of attachment is important for forward motion, how do the parasites lacking TgMIC2 move at normal speeds in the absence of constrictions? It is possible that both the wild-type and mutant parasites generate small forces distributed over their entire surface (in addition to larger forces generated at the constriction in wildtype) that are collectively sufficient to drive motility but individually below our level of detection. Alternatively, the loss of TgMyoA or TgMIC2 might lead to compensatory changes in the expression of other genes with overlapping or redundant function [23, 48, 49]. The TgMIC2 KO parasites do in fact show changes in the expression of several other microneme and motility-associated proteins [31]. Perhaps most interestingly, parasites lacking

TgMyoA and/or TgMIC2 might use a motility mechanism that is entirely different from that of wild-type parasites (*e.g.*, [18, 22]), does not involve a constriction, and is not as efficient as the wild-type mechanism but is nevertheless capable of supporting motility and sustaining the parasite's lytic cycle. By analogy with observations from animal cells [50], parasites might use different mechanisms for motility in different situations such as squeezing through tight junctions [8] *vs.* migrating through loose interstitial tissues. Any attempt to develop new therapeutics targeting parasite motility [51, 52] will need to take into account the possibility that the parasite has multiple mechanisms to draw upon. If the motility of the mutants is driven by a different mechanism than that of wild-type parasites, higher sensitivity 3D traction force mapping may reveal informative differences in their pattern of force generation.

A guidance system for motility? The forward motion of the TgMIC2 knockout parasites, which lack the circular attachment zone, was significantly more disorganized than the motility of wild-type parasites. The mutant parasites could still generate relatively long trajectories, but the trajectories took them less far from their starting point than wild-type parasites. As discussed above, attachment to the matrix via a circular ring of adhesion likely contributes to the rotational twisting of the parasite as it moves forward and thereby to the overall helicity of the parasite's trajectory. Extensive studies of the helical swimming behavior of bacteria has shown that a curved cell shape and helical trajectory are a particularly efficient way for small cells to move through viscous media [53-57]. Engagement of *T. gondii* with the extracellular matrix through a circular band of adhesion may therefore not only create a fixed platform for the parasite to pull against and move forward; it may also function as a guidance system to help the parasite move efficiently through the various environments it encounters as it disseminates through the tissues of the unfortunate organisms it infects.

Methods

Cell Culture. Parasites were propagated by serial passage in human foreskin fibroblasts (HFFs). HFFs were grown to confluence in Dulbecco's Modified Eagle's Medium (DMEM) (Life Technologies, Carlsbad, CA) containing 10% (vol/vol) heat-inactivated fetal bovine serum (FBS) (Life Technologies, Carlsbad, CA), 10 mM HEPES pH 7, and 100 units/ml penicillin and 100 ug/ml streptomycin, as previously described [58]. Prior to infection with *T. gondii*, the medium was changed to DMEM supplemented with 10mM HEPES pH 7, 100 units/ml penicillin and 100 ug/ml streptomycin, and 1% (vol/vol) FBS.

Pitta Chamber Assembly. Coverglasses (22×22 and 24×50 mm) were washed Alconox detergent, rinsed with tap water, deionized water, and ethanol (100%), and air dried. Two strips of double-sided tape (Scotch 3M, St. Paul, MN) were placed 3 mm apart on the large coverglass, and the small coverglass was placed on top of the tape and pressed firmly to ensure a complete seal. The flow cell volume was approximately 10 µl.

Matrigel Matrix. Pitta chambers containing parasites embedded in polymerized Matrigel were prepared as previously described [11]. Briefly, parasites were harvested from infected HFFs via syringe release and passed through a 3 µm Nucleopore filter (Whatman, Piscataway NJ). Parasites were pelleted and resuspended at in Live Cell Imaging Solution (LCIS) buffer containing 0.5 mg/ml Hoechst 33342 (Thermo Scientific, Waltham, MA). Hoechst 33342 was allowed to stain the parasites for 10 minutes at room temperature before the parasites were mixed with Matrigel and LCIS on ice in a 1:3:3 (vol/vol/vol) ratio and immediately added to a Pitta chamber. The Pitta chamber was incubated for three minutes at 35°C in a custom-build heated microscope enclosure (UVM Instrumentation and Model Facility, Burlington, VT) before imaging.

Fibrin Matrix. Unlabeled and Alexa-Fluor 647-labeled fibrinogen (both from Thermo Scientific, Waltham, MA) were each dissolved in 0.1M sodium bicarbonate (pH 8.3) to 15 mg/ml. Aliquots of 50 µl were flash frozen and stored at -80C until use. Fibrin gels [32] containing parasites were prepared in Pitta chambers as follows. The parasite culture medium was replaced with LCIS buffer before harvesting via syringe release and filtering. 500 µl of the parasite suspension were pelleted and resuspended in LCIS buffer to achieve a higher parasite concentration. Parasites were mixed with fibrinogen (final fibrinogen concentration of 2.25, 4.5, or 9 mg/ml). LCIS buffer containing thrombin (Sigma, Burlington, MA) and FBS was added to the parasite-fibrinogen suspension for final concentrations in the ~15ul volume Pitta chambers of $1.5x10^8$ parasites per ml, 1 unit of thrombin, and 10% FBS. The mixture was immediately pipetted into a Pitta chamber and allowed to polymerize at room temperature for three minutes before mounting on the microscope and imaging.

Image Acquisition. A Nikon Eclipse TE300 epifluorescence microscope (Nikon Instruments, Melville, NY) equipped with a NanoScanZ piezo Z stage insert (Prior Scientific, Rockland, MA) was used to image parasites. See Table 1 for details of the imaging setup. Time-lapse video stacks were collected with an iXON Life 888 EMCCD camera (Andor Technology, Belfast, Ireland) using NIS Elements software v.5.11 (Nikon Instruments, Melville, NY). Fluorescently labeled parasites were imaged using a pE-4000 LED illuminator (CoolLED, Andover England) and a 89402 Quad filter (Chroma, Bellows Falls, VT).

Stacks consisting of individual images (1024 pixel× 384 pixel) captured 0.5-1 µm apart in *z*, covering a total of 10-40 µm, were collected using either a 20× PlanApo λ (0.65 pixel/µm, NA 0.75) or 60 × PlanApo λ (0.22 pixel/µm, NA 1.4) objective as described in Table 1. The same volume was successively imaged 60-360 times over the course of 77-302 seconds. The camera was set to trigger mode, no binning, readout speed of 35 MHz, conversion gain of 3.8x, and EM gain setting of 300.

Table 1: Imaging parameters for the different experiments described

Experiment	Objective	Fluorochrome (Excitation/emission wavelengths)	Image spacing in *z*	Exposure time per image	Number of Image stacks	Total time	Volume (*x, y, z*)
Microspheres (Matrigel)	60×	DragonGreen (490/507-530nm)	41 slices, 1µm apart	16 ms	60	77s	225.3 µm × 84.5 µm × 40 µm
		tdTomato parasites (550/579-608 nm)					
Fibrin vs Matrigel and TgMIC2 KO directionality	20×	Hoechst 33342 (385/420-449 nm)	41 x 1 µm	16 ms	120	80s	665.6 µm × 249.6 µm × 40 µm
Force Mapping, WT (Fibrin)	60×	tdTomato parasites (550/579-608 nm)	50 x 0.5 µm	16 ms	60	96s	225.3 µm × 84.5 µm × 24.5 µm
		Fibrin (635/666-723 nm)					
WT, TgMyoA KO, TgMIC2 KO; Brightfield (Matrigel, fibrin)	60×	N/A: Brightfield	21 x 1 µm	40 ms	360	302s	225.3 µm × 225.3 µm × 20 µm
Nuclear size vs constriction (Matrigel)	20×	Hoechst 33342 (385/420-449 nm)	41 x 1 µm	16 ms	60	80s	665.6 µm × 249.6 µm × 40 µm
		Anti-SAG1 Alexa 548 (550/579-608 nm)					
	60×	Hoechst 33342		16 ms	60	96s	

TgMyoA KO Force Map (Fibrin)		(385/420-449 nm)	50 x 0.5 µm				225.3 µm × 84.5 µm × 24.5 µm
		Fibrin (635/666-723 nm)					
TgMIC2 KO Force Map (Fibrin)	60×	YFP cytosol (490/507-530 nm)	50 x 0.5 µm	16 ms	60	96s	225.3 µm × 84.5 µm × 24.5 µm
		Fibrin (635/666-723 nm)					

Tracking Parasite Motility. Datasets were analyzed in Imaris ×64 v. 9.2.0 (Bitplane AG, Zurich, Switzerland). Fluorescently labeled parasite nuclei were tracked using the ImarisTrack module within a 1018 pixel × 380 pixel region of interest to prevent artifacts from tracking objects near the border. Spot detection used an estimated spot diameter of 3.0 × 3.0 × 6.0 µm (*x, y, z)*. A maximum distance of 6.0 µm and a maximum gap size of 2 frames were applied to the tracking algorithm. Tracks with durations under 10 seconds or displacements of less than 2 µm were discarded to avoid tracking artifacts and parasites moving by Brownian motion, respectively [11]. Accurate tracking was confirmed by visual inspection of parasite movements superimposed on their calculated trajectories.

For the comparison between Matrigel and fibrin, we analyzed three biological replicates, each consisting of three technical replicates. Student's T-tests were used to determine statistical significance between fibrin and Matrigel samples.

Fibrin Deformation with FIDVC. The Fast Iterative Digital Volume Correlation (FIDVC) algorithm was used to calculate 3D fibrin displacements [33] by comparing two consecutive image volumes. The image volumes were cropped to 384 × 384 pixels (*x,y)* × 48 slices (*z*) before running FIDVC. The initial interrogation window size was set to 32 × 32 × 32 and the program was run incrementally. The displacements arrows were plotted using a combination of previously described MATLAB code for 2D quiver color-coded for directionality [32] and custom MATLAB code. To filter out background signal, we calculated the mean magnitude of the 16,807 displacement vectors for each timepoint comparison in each dataset. The displacement detection threshold for that dataset was set as three standard deviations above the lowest of these mean values.

Rheology. A Lumicks C-Trap laser trap was used to determine the viscoelasticity of the fibrin. First, the power density spectrum (PDS) was calculated with 0.91 µm styrene beads in a flow cell. The beads were embedded in a fibrin gel, and a bead was captured in the laser trap. The flow cell was oscillated on the

stage in the *y* dimension at different frequencies (1, 5, 10, 20, 50, 100 Hz), each for 5 seconds, at a fixed amplitude of 100 nm. The trap position and force in the *y* dimension were captured at 78 kHz. The elastic (in-phase) and viscous (out-of-phase) moduli were calculated using the bead's force trace. The data were analyzed in R studio. A sliding two-sided window filter, size 78, was applied to both traces. The position signal was fitted with the function $position = A * \sin(t * f - phase)$ using nonlinear least-squares with fit parameters for amplitude (A), frequency (f), and phase. The force readout was fit with the function $force = B * \sin(t * f - phase) + C * \cos(t * f - phase)$ using nonlinear least-squares with fitting parameters B and C, where B is the in-phase (elastic) component of the force. The C fit parameter divided by the velocity of the trap equals the viscous modulus.

Confocal microscopy. A Nikon A1R-ER point-scanning confocal microscope was used to image the fluorescent fibrin gels. Using Galvano scanning, image (1024 × 1024 pixel) stacks were captured with 60× Apo (0.1 µm/pixel, NA 1.49l) objective with 0.2 µm spacing over 5 µm. The LUNV laser was used at wavelength 633 nm and the pinhole size was 28.10 µm.

Figure legends

Figure 1: Parasites moving in 3D deform the surrounding Matrigel. **(A)** Sequential time series images in a single *z*-plane demonstrate that Dragon Green-labeled microspheres embedded within the matrix are displaced towards moving tdTomato-expressing parasites; note displacement of the green bead closest to the fixed crosshair in the merged images. See Video 1 for the entire time series, and Video 2 for a second example of bead displacement by a moving parasite. (C) Brightfield images showing a constriction progressing from the anterior to the posterior end of a parasite as the parasite moves forward one body length. Scale bars = 5 µm, timestamps in seconds.

Figure 2: 3D traction force mapping in fluorescent fibrin reveals that the matrix is periodically pulled in towards the constriction during parasite motility. **(A)** Sequential time series images in a single *z*-plane of a tdTomato-expressing parasite moving in fibrin (boxed). Scale bar = 10 µm, timestamps in seconds. **(B)** Force maps from the corresponding *z* subvolume plane of the data shown in (A); each map depicts the motions of the matrix between the two consecutive timepoints, as indicated by the brackets. Arrow length (multiplied 24-fold for display) indicates the magnitude of matrix displacement and arrow color the directionality (see color wheel). **(C)** Zoomed images showing the force maps from the boxed region of panel B, after background subtraction (see text), overlaid on the parasite images from panel A. Length of arrows indicating displacement magnitude are multiplied 15-fold for display. Note inward displacement vectors of the matrix in the second panel, outward displacement vectors (relaxation)

of the matrix in the third panel, and no detectable displacement vectors in the fourth panel even though the parasite continues to move. **(D)** Zoomed overlays of the force maps (background subtracted) and images of a moving parasite from a second dataset. The inwardly displaced matrix in this example did not relax back to its original position within the time frame of the experiment. Arrow lengths indicating displacement magnitude are multiplied 15-fold for display. Scale bars in C and D = 5 µm. Empty red arrowheads indicate position of the constriction.

Figure 3: The constrictions are tightly linked with motility and are sufficiently narrow to deform the parasite nucleus as it passes through. (A) The time interval between constrictions during motility of individual parasites (n=188). A negative time interval (black bars) corresponds to the presence of two constrictions in the same parasite for the indicated amount of time. Green bars denote back-to-back constrictions and grey bars denote a delay between constrictions for the indicated amounts of time. In 27.1% of the constrictions (not plotted), no subsequent constriction occurred within the time frame of the experiment. **(B)** Example of a parasite with two overlapping constrictions. Brightfield images; scale bar = 5 µm; timestamps in seconds. **(C)** Number of constrictions observed *vs*. distance traveled, expressed in terms of binned parasite body lengths (*i.e.*, multiples of 7 µm). **(D)** Width of non-constricting parasites halfway along their longitudinal axis *vs.* width at the constriction halfway along the parasite's longitudinal axis. **(E)** Dimensions of the nucleus (as defined by Hoechst 33342 staining) in non-constricting parasites *vs*. nuclei passing through a constriction. Length is parallel and width perpendicular to the long axis of the parasite. Samples were compared by Student's two-tailed t-test.

Figure 4: Knockout of TgMIC2 results in the loss of the constriction and less directional motility. (A) Brightfield images showing a moving TgMIC2 knockout parasite without a constriction. Scale bar = 2.5 µm, timestamps in seconds. **(B)** Representative plots of nuclear shape (ratio of the nuclear diameter perpendicular *vs.* parallel to the long axis of the parasite) in one wildtype and one TgMIC2 knockout parasite (black circles and green squares, respectively), as the parasites moved over time. The wild-type parasite underwent multiple concurrent constrictions along its 15 µm trajectory. For the combined data from multiple parasites, see Suppl. Figure 9A. **(C)** Ratio of parasite displacement (D) to trajectory length (L) for wildtype (n= 1399) and TgMIC2 knockout (n= 165) parasites (black circles and green squares, respectively). The calculated means (± SD) are from three independent biological replicates; see Suppl. Figure 9B. In panels (B and C), "wildtype/WT" refers to the TgMIC2 LoxP parasites [31] before treatment with rapamycin to excise *MIC2*.

Supplemental figure legends

Suppl. Figure 1: Schematic illustrations of the glideosome. In the linear motor model of motility, the TgMyoA motor (TgMyoA and its associated light chains, TgMLC1 and either TgELC1 or TgELC2) is anchored to the parasite's inner membrane complex (IMC) via the acylated protein TgGAP45 and the transmembrane proteins TgGAP40 and TgGAP50. The lumenal portion of GAP50 is thought to interact with GAPM, a protein that spans the inner IMC membrane and likely connects the entire glideosome to the underlying parasite cytoskeleton. Short actin filaments located between the parasite plasma membrane and the IMC are connected to ligands on the substrate through a linker protein, possibly GAC, which binds to the cytosolic tails of surface adhesins such as TgMIC2. The TgMyoA motor displaces the actin filaments rearward; because the motor is connected to the IMC and the actin is connected to the substrate, this causes the parasite to move forward relative to the substrate. Modified from [26] and re-used here under the terms of a Creative Commons Attribution 4.0 International license (doi/10.1128/mSphere.00823-20).

Suppl. Figure 2: **Comparison of parasite motility in fluorescent fibrin *vs.* Matrigel. (A)** Confocal image of a 2.25 mg/ml fluorescent fibrin gel; scale bar = 10 µm. **(B)** Maximum intensity projections of tdTomato-expressing wildtype parasites moving in 3D in Matrigel (top) *vs.* fibrin (bottom). Scale bar = 40 µm. **(C)** Comparison of the maximum and mean speeds of parasites in 2.25 mg/ml fibrin or Matrigel. Horizontal bars indicate mean (± SD) from 3 independent biological replicates (Matrigel n=853, Fibrin n=913). $p > 0.05$ for both maximum and mean speed, Student's two-tailed t-test. **(D)** A parasite moving in fluorescent fibrin (see Video 4) visibly deforms the matrix, as evident from the non-coincident fluorescence signals at two consecutive time points (merge). Scale bar = 10 µm, timestamps in seconds.

Suppl. Figure 3: **Determining threshold of detection in 3D traction force mapping.** The plots show all *x-y* vector magnitudes calculated by FIDVC for representative image volume datasets from (A) wild-type parasites, (B) no parasites (fluorescent fibrin only), (C) TgMyoA knockout parasites, and (D) TgMIC2 knockout parasites. Each comparison between two consecutive timepoints results in 16,807 vectors; panel A also shows an expanded plot of the 16,807 datapoints for T6-T7 (no constricting parasites in the field) and T7-T8 (one constricting parasite in the field). For each time series, the comparison between the two consecutive timepoints that gave the lowest mean displacement magnitude was used to set the background level: any displacements less than three standard deviations above this mean were considered noise for that time series.

Suppl. Figure 4: **Rheological properties of the fibrin matrix.** The viscoelastic properties of the fibrin matrix were measured by laser trapping of 0.91 µm styrene beads. **(A)** The laser trap position at 1 Hz (top panel) and the resulting force as measured by the laser trap (bottom), showing a primarily in-phase force

response. **(B)** The laser trap position and output force at 1-20Hz were used to calculate the elastic and viscous properties of the matrix; these frequencies were chosen to encompass the approximate speed of a moving parasite. Bars indicate mean (± SD).

Suppl. Figure 5: All displacement vectors point in towards a moving parasite. Matrix displacement vectors from all subvolumes (49 (*x)* x 49 (*y*) X 7 (*z*)) in the image volume were calculated by FIDVC and projected onto the *x-y, x-z* and *y-z* planes, as indicated (axis labels are in µm). Length of arrows indicating displacement magnitude are multiplied 10-fold for display. The data correspond to the parasite shown in the second panel of Fig. 2C and frame 2 of video 6. The moving parasite is outlined on each z slice with red circles, and the position of the constriction is indicated by either an empty arrowhead (*x-y*) or a black dot (*y-z*).

Suppl. Figure 6: **Pattern of pulling, holding and releasing the matrix during individual constriction events.** The number of consecutive frames in which: the matrix displaced in towards the parasite (pull); no further matrix displacement was observed (hold); and the matrix moved away from the parasite towards its original position (release) are indicated. Total number of constrictions analyzed = 25.

Suppl. Figure 7: **Additional examples showing that matrix displacement is directed primarily in towards the constriction in moving parasites.** Empty red arrowheads indicate position of the constriction and white arrows the direction of parasite travel. Length of arrows indicating displacement magnitude are multiplied 15-fold for display. Scale bars = 5 µm.

Suppl. Figure 8: **The small number of TgMyoA knockout parasites that move more than one body length show no evidence of constrictions and produce no detectable force on the fibrin matrix. (A)** Far fewer TgMyoA knockout parasites move more than one body length compared to wild-type parasites (*i.e.,* LoxP TgMyoA parasites before rapamycin treatment). **(B)** Brightfield images of a right-angled "stairstep" turn made by a moving TgMyoA knockout parasite. Scale bars = 5 µm, timestamps in seconds. **(C-E)** Sequential fluorescence images in a single *z*-plane of a moving TgMyoA knockout parasite stained with Hoechst 33342 (to stain the parasite nucleus), the corresponding force maps, and the zoomed images (panel E) showing the force maps from panel D (boxed region), with background subtraction, overlaid on the parasite images from panel C. The parasite continued to move for another 24 sec; no signal above background was seen on the force maps at any time along its trajectory. Length of arrows indicating displacement magnitude are multiplied 24-fold in (C) and 15-fold in (D) for display. Scale bar = 5 µm, timestamps in seconds.

Suppl. Figure 9: **TgMIC2 knockout parasites show no evidence of constrictions, produce no detectable force on the fibrin matrix and move less directionally. (A)** Ratio of nuclear diameter

perpendicular *vs.* parallel to the long axis of the parasite in moving wild-type (n=9) and TgMIC2 knockout (n=10) parasites over time. The lower ratios (~0.5) seen in wild-type parasites correspond to time points during which the nuclei were squeezing through a constriction. **(B)** Ratio of parasite displacement to trajectory length for wild-type and TgMIC2 knockout parasites (three biological replicates, each consisting of three technical replicates). The horizontal bars indicate means (+/- SD), which were compared by Student's two-sided t-test. Note that in panels A and B, "wildtype/WT" refers to the TgMIC2 LoxP parasites [31] before treatment with rapamycin to excise *MIC2*. **(C-E)** Sequential fluorescence images in a single *z*-plane of a moving TgMIC2 knockout parasite expressing YFP, the corresponding force maps, and the zoomed images (panel E) showing the force maps from panel D (boxed region), with background subtraction, overlaid on the parasite images from panel C. The parasite continued to move for another 18 sec; no signal above background was seen on the force maps at any time along its trajectory. Length of arrows indicating displacement magnitude are multiplied 24-fold in (C) and 15-fold in (D) for display. Scale bars = 10 µm (panel C), 5 µm (panel E); timestamps in seconds.

Acknowledgements. This work was supported by U.S. Public Health Service grants: AI139201 and AI137767 (G.E.W): GM141743 and S10OD026884 for the Lumicks C-Trap (D.M.W); and T32AI055402 and F31AI145214 (R.V.S.), as well as American Heart Association grant 19PRE34370071 (R.V.S.) We thank Nicole Bouffard and Dr. Douglas Taatjes of the UVM Larner College of Medicine Microscopy Imaging Center (RRID# SCR_018821) for helpful advice and assistance with the Nikon A1R-ER confocal microscope, which is supported by U.S. Public Health Service grant 1S10OD025030-01 from the National Center for Research Resources. We thank: Dr. Markus Meissner for sharing the TgMyoA and TgMIC2 knockout parasites; Dr. David Sibley for anti-SAG1 antibody; Drs. Mark Rould, Alex Dunn, Christina Hueschen, Li-av Segev Zarko, John Boothroyd, Leanna Owen, Markus Meissner, and Ulrich Schwarz for helpful discussions; Anne Snyder, Drs. Robyn Kent, Christina Hueschen, and Chris Huston for helpful feedback on the manuscript. Finally, we thank Dr. Christian Franck for making the FIDVC code used in this work freely available through GitHub.

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Figure 1

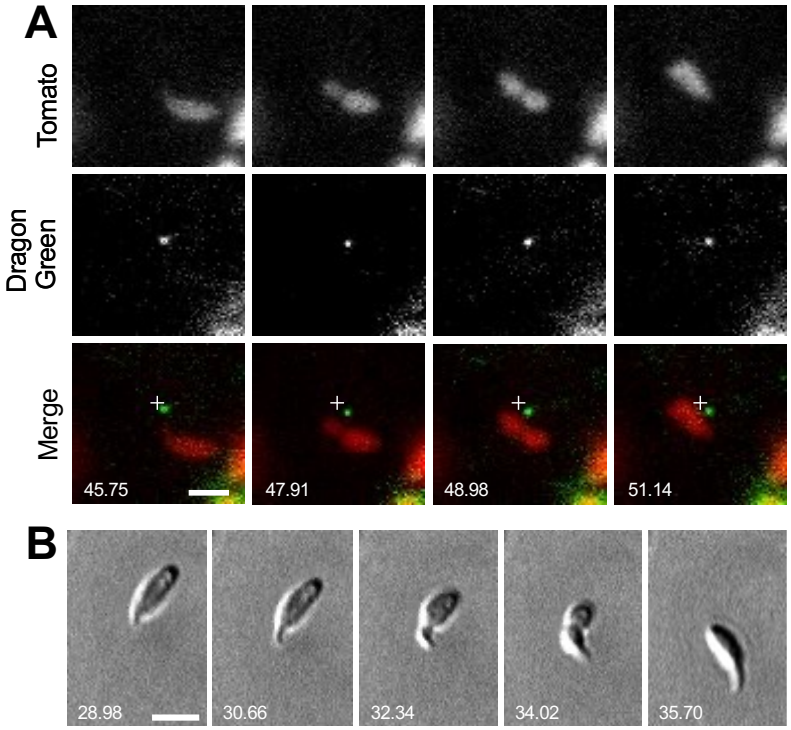

A
Tomato
Dragon Green
Merge
45.75
47.91
48.98
51.14
B
28.98
30.66
32.34
34.02
35.70

Figure 2

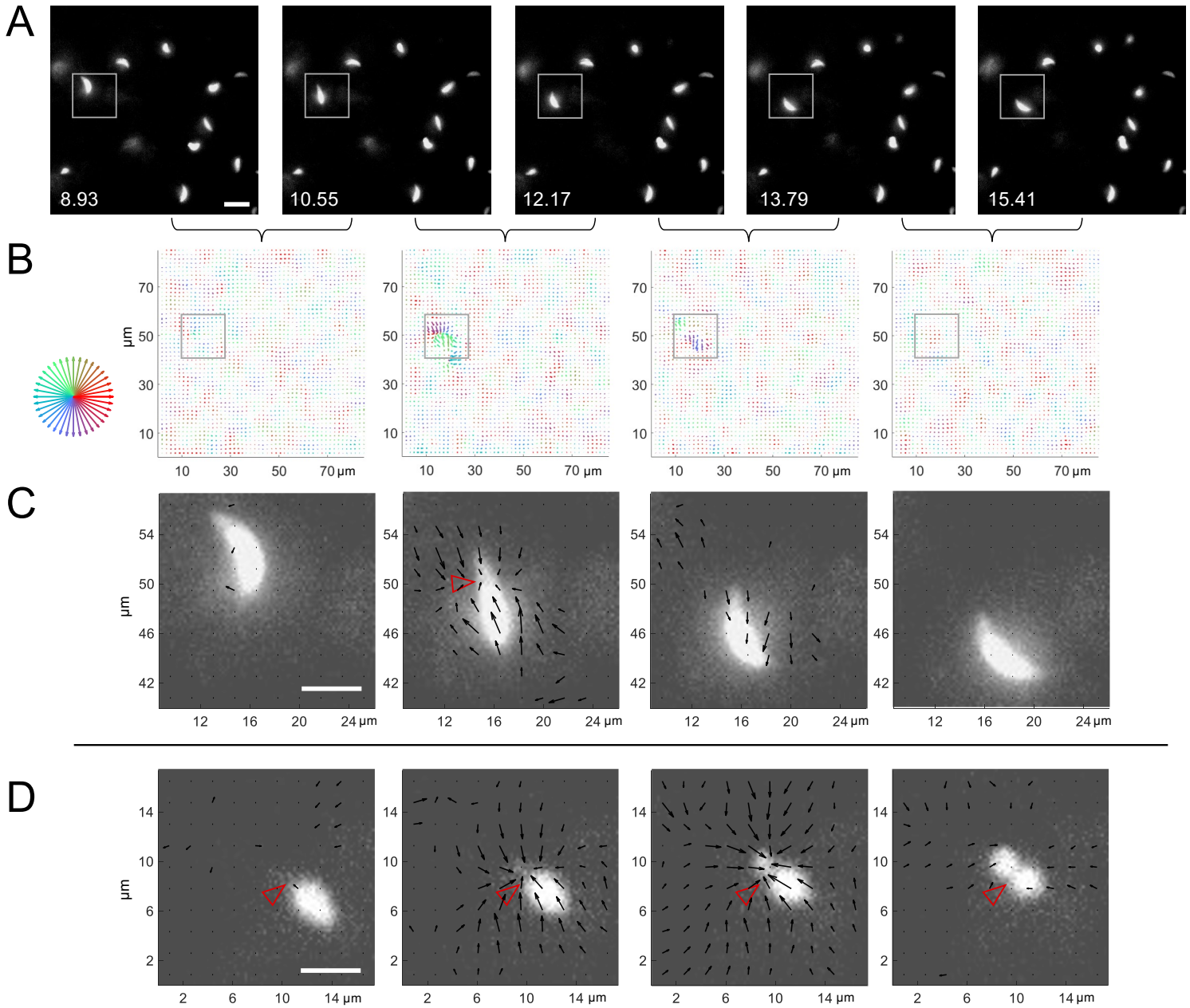


Figure 3

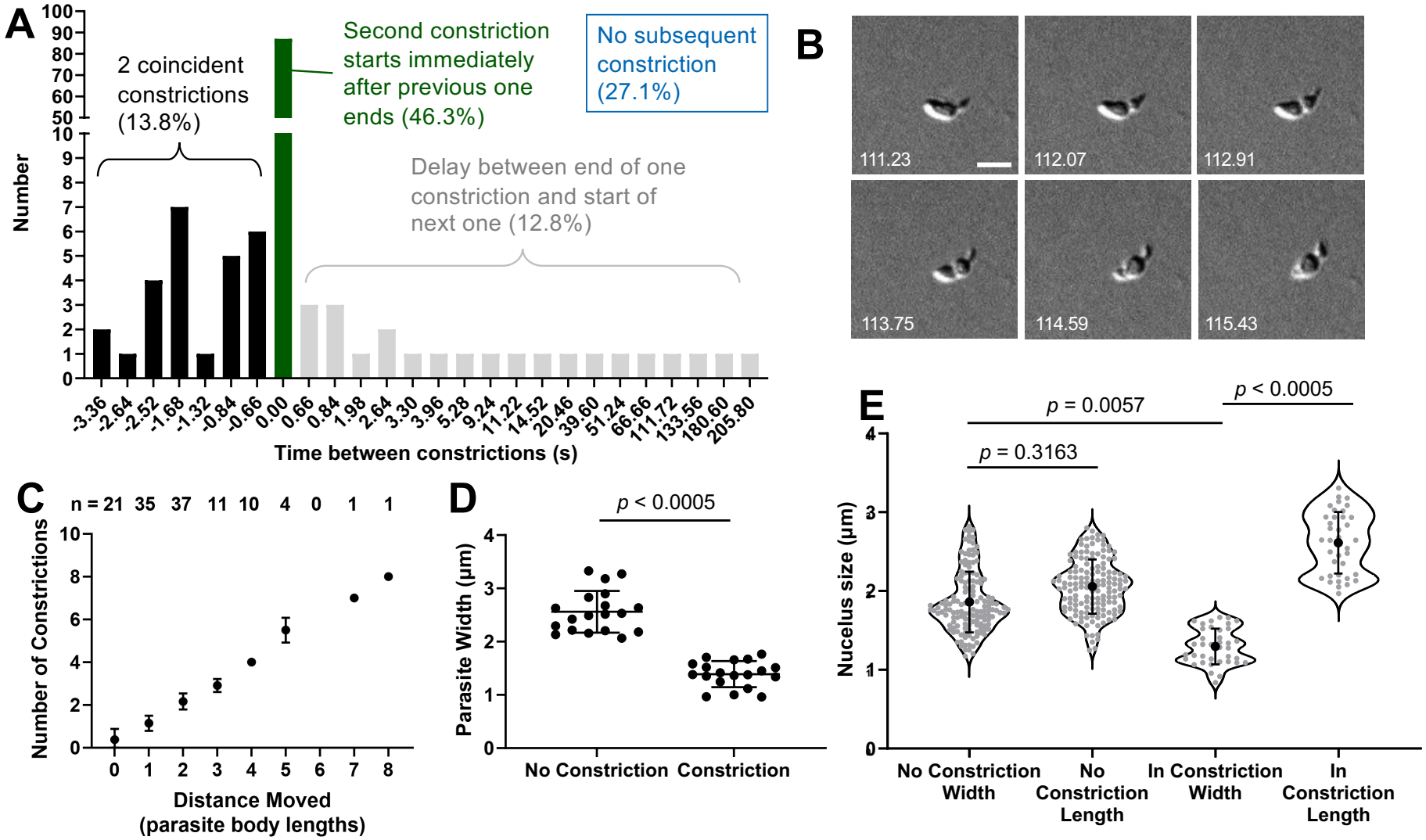
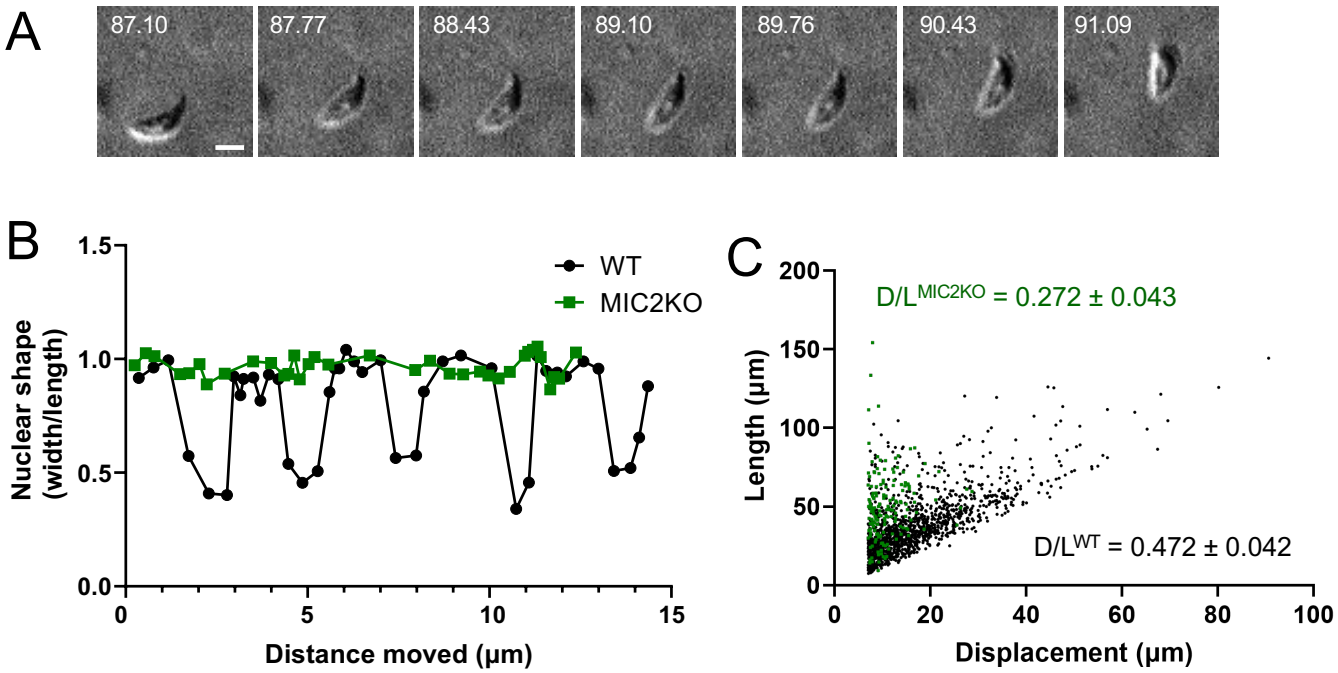

A
2 coincident constrictions (13.8%)
Second constriction starts immediately after previous one ends (46.3%)
No subsequent constriction (27.1%)
Delay between end of one constriction and start of next one (12.8%)
Number
Time between constrictions (s)
-3.36 -2.64 -2.52 -1.68 -1.32 -0.84 -0.66 0.00 0.66 0.84 1.98 2.64 3.30 3.96 5.28 9.24 11.22 14.52 20.46 39.60 51.24 66.66 111.72 133.56 180.60 205.80
B
111.23
112.07
112.91
113.75
114.59
115.43
C
n = 21 35 37 11 10 4 0 1 1
Number of Constrictions
Distance Moved (parasite body lengths)
D
p < 0.0005
Parasite Width (µm)
No Constriction
Constriction
E
p = 0.0057
p < 0.0005
p = 0.3163
Nucelus size (µm)
No Constriction Width
No Constriction Length
In Constriction Width
In Constriction Length

Figure 4


A
87.10
87.77
88.43
89.10
89.76
90.43
91.09
B
WT
MIC2KO
Nuclear shape (width/length)
1.5
1.0
0.5
0.0
0
5
10
15
Distance moved (μm)
C
D/L^MIC2KO = 0.272 ± 0.043
D/L^WT = 0.472 ± 0.042
Length (μm)
200
150
100
50
0
0
20
40
60
80
100
Displacement (μm)