

This is a repository copy of *Role for the flagellum attachment zone in Leishmania anterior cell tip morphogenesis*.

White Rose Research Online URL for this paper:

<https://eprints.whiterose.ac.uk/167166/>

---

**Article:**

Halliday, Clare, Yanase, Ryuji, Catta-Preta, Carolina Moura Costa et al. (6 more authors) (2020) Role for the flagellum attachment zone in Leishmania anterior cell tip morphogenesis. PLOS PATHOGENS. ISSN 1553-7366

---

**Reuse**

Items deposited in White Rose Research Online are protected by copyright, with all rights reserved unless indicated otherwise. They may be downloaded and/or printed for private study, or other acts as permitted by national copyright laws. The publisher or other rights holders may allow further reproduction and re-use of the full text version. This is indicated by the licence information on the White Rose Research Online record for the item.

**Takedown**

If you consider content in White Rose Research Online to be in breach of UK law, please notify us by emailing [eprints@whiterose.ac.uk](mailto:eprints@whiterose.ac.uk) including the URL of the record and the reason for the withdrawal request.

1 **Role for the flagellum attachment zone in *Leishmania* anterior cell tip morphogenesis**

2

3 Clare Halliday<sup>1</sup>, Ryuji Yanase<sup>1,2</sup>, Carolina Moura Costa Catta-Preta<sup>3</sup>, Flavia Moreira-Leite<sup>1</sup>, Jitka

4 Myskova<sup>4</sup>, Katerina Pruzinova<sup>4</sup>, Petr Volf<sup>4</sup>, Jeremy C. Mottram<sup>3</sup> and Jack D. Sunter<sup>1\*</sup>

5

6 <sup>1</sup>Department of Biological and Medical Sciences, Oxford Brookes University, Oxford, UK

7 <sup>2</sup>Shimoda Marine Research Center, University of Tsukuba, Shizuoka, Japan

8 <sup>3</sup>York Biomedical Research Institute and Department of Biology, University of York, York, UK

9 <sup>4</sup>Department of Parasitology, Charles University, Prague, Czech Republic

10 \*corresponding author: jsunter@brookes.ac.uk

11

12

13 **Abstract**

14 The shape and form of the flagellated eukaryotic parasite *Leishmania* is sculpted to its ecological  
15 niches and needs to be transmitted to each generation with great fidelity. The shape of the *Leishmania*  
16 cell is defined by the sub-pellicular microtubule array and the positioning of the nucleus, kinetoplast  
17 and the flagellum within this array. The flagellum emerges from the anterior end of the cell body  
18 through an invagination of the cell body membrane called the flagellar pocket. Within the flagellar  
19 pocket the flagellum is laterally attached to the side of the flagellar pocket by a cytoskeletal structure  
20 called the flagellum attachment zone (FAZ). During the cell cycle single copy organelles duplicate with  
21 a new flagellum assembling alongside the old flagellum. These are then segregated between the two  
22 daughter cells by cytokinesis, which initiates at the anterior cell tip. Here, we have investigated the  
23 role of the FAZ in the morphogenesis of the anterior cell tip. We have deleted the FAZ filament protein,  
24 FAZ2 and investigated its function using light and electron microscopy and infection studies. The loss  
25 of FAZ2 caused a disruption to the membrane organisation at the anterior cell tip, resulting in cells  
26 that were connected to each other by a membranous bridge structure between their flagella.  
27 Moreover, the FAZ2 null mutant was unable to develop and proliferate in sand flies and had a reduced  
28 parasite burden in mice. Our study provides a deeper understanding of membrane-cytoskeletal  
29 interactions that define the shape and form of an individual cell and the remodelling of that form  
30 during cell division.

31

32 **Author summary**

33 *Leishmania* are parasites that cause leishmaniasis in humans with symptoms ranging from mild  
34 cutaneous lesions to severe visceral disease. The life cycle of these parasites alternates between the  
35 human host and the sand fly vector, with distinct forms in both. These different forms have different  
36 cell shapes that are adapted for survival in these different environments. *Leishmania* parasites have  
37 an elongated cell shape with a flagellum extending from one end and this shape is due to a protein

38 skeleton beneath the cell membrane. This skeleton is made up of different units one of which is called  
39 the flagellum attachment zone (FAZ), that connects the flagellum to the cell body. We have found that  
40 one of the proteins in the FAZ called FAZ2 is important for generating the shape of the cell at the point  
41 where the flagellum exits the cell. When we deleted FAZ2 we found that the cell membrane at the  
42 end of the cell was distorted resulting in unusual connections between the flagella of different cells.  
43 We found that the disruption to the cell shape reduces the ability of the parasite to infect mice and  
44 develop in the sand fly, which shows the importance of the parasite shape.

45

## 46 Introduction

47 The kinetoplastid parasites have a defined shape and form, which varies during their life cycle  
48 depending on the specific ecological niche. These different shapes and forms of *Leishmania spp* and  
49 *Trypanosoma brucei* are determined by their highly organised sub-pellicular microtubule array.  
50 Different forms can be categorised based on the relative positions of three key structures:- i) the  
51 nucleus, ii) the kinetoplast (the condensed mitochondrial DNA) and iii) the flagellum and its associated  
52 flagellar pocket (an invagination of the cell membrane at the base of the flagellum) [1]. The flagellar  
53 pocket is a critical feature as it is the site of all endo- and exocytosis in these parasites [2].

54 The *Leishmania* flagellar pocket consists of two regions, the bulbous lumen and the flagellar pocket  
55 neck (Fig 1A). At the distal end of the bulbous lumen is the flagellar pocket collar, a cytoskeletal  
56 structure that cinches in the cell membrane to form this bulbous domain [3]. Distal to this point, within  
57 the flagellar pocket neck region, the cell body membrane is closely apposed to the flagellum  
58 membrane until the flagellum exits the cell body. Since the *Leishmania* promastigote flagellum  
59 extends from the anterior cell tip it has traditionally been described as 'free'; however, the basal  
60 region is firmly attached to the cell body within the flagellar pocket neck region [3].

61 **Figure 1.** FAZ2 null mutant *Leishmania* parasites grew slower and had flagella-to-flagella connections.  
62 (A) Schematic of *Leishmania* promastigote flagellar pocket from two different angles, giving a side-on  
63 and plan view of the flagellum attachment zone (FAZ). The flagellar pocket is divided into two regions  
64 (bulbous and neck) with the microtubule quartet (MtQ) wrapping around the bulbous region of the  
65 flagellar pocket before terminating in the neck region. The different FAZ domains and proteins present  
66 in these domains are shown. (B) Light micrographs of parental, FAZ2 null mutant and FAZ2 add back  
67 cells expressing the flagellum membrane protein SMP1 tagged with eGFP-Ty (green) and the DNA is  
68 stained with Hoechst 33342 (cyan). FAZ2 add back cells were also expressing mChFP::FAZ2 (magenta).  
69 Scale bar is 5  $\mu$ m. (C) Growth curve of the parental, FAZ2 null mutant and FAZ2 add back cells over a  
70 72 h time period. The mean  $\pm$  s.d. from 3 independent experiments is plotted. (D) Light micrographs

71 of cell types observed in culture. Scale bar is 5  $\mu\text{m}$ . (E) Quantitation of cell types seen in culture for  
72 parental, FAZ2 null mutant and FAZ2 add back cells. The mean  $\pm$  s.d. from 3 independent experiments  
73 is plotted. For each experiment  $\geq 91$  cells were counted. (F) Cell cycle category counts for parental,  
74 FAZ2 null mutant and FAZ2 add back cells. F – flagellum, K – kinetoplast, N – nucleus, F to F – two cells  
75 connected via their flagella. The mean  $\pm$  s.d. from 3 independent experiments is plotted. For each  
76 experiment  $\geq 163$  cells were counted. (G) Light micrographs of FAZ2 null mutant cells expressing  
77 SMP1::eGFP-Ty showing the phase and SMP1::eGFP-Ty channels, with an example of two FAZ2 null  
78 mutant cells connected via their flagella and of a one flagellum FAZ2 null mutant cell that had a  
79 residual structure on the flagellum near the anterior cell tip (white arrow). Scale bar is 5  $\mu\text{m}$ .

80 The attachment of the flagellum to the cell body is mediated by the flagellum attachment zone (FAZ),  
81 a complex structure, which contains many cytoskeletal elements [4,5]. The FAZ connects the cell body  
82 cytoskeleton to the cytoskeleton of the flagellum through the cell body and flagellum membrane and  
83 consists of three major domains:- i) the cell body domain, ii) the intermembrane domain, and iii) the  
84 flagellum domain [5] (Fig 1A). The *T. brucei* FAZ is much longer than the *Leishmania* equivalent and  
85 has been the subject of more extensive study. In *T. brucei* electron microscopy studies have defined  
86 the individual cytoskeletal elements that together form the FAZ. Within the cell body domain there is  
87 the FAZ filament that runs parallel to the microtubule quartet (a specialised set of four microtubules)  
88 that together form a seam within the sub-pellicular microtubule array [5,6]. From the FAZ filament  
89 and microtubule quartet a set of fibres extend to a linear array of regular junctional complexes  
90 embedded in the cell body membrane. These junctional complexes connect across to the flagellum  
91 membrane and together with that membrane form the intermembrane domain. Another set of  
92 intraflagellar filaments then constitute the linker structure that connects to the proximal domain of  
93 the paraflagellar rod (an extra-axonemal structure) [7].

94 Work in many labs and more recently the genome-wide tagging project, TrypTag has identified many  
95 FAZ proteins in *T. brucei* [8–15]. The function and interaction of a number of these proteins has been

96 investigated; for example, the depletion of FAZ2, a cell body FAZ domain protein resulted in full length  
97 flagellum detachment and the loss of other cell body FAZ domain proteins such as FAZ1 and FAZ8 [13].  
98 The *Leishmania* orthologs of many *T. brucei* FAZ proteins localised to the *Leishmania* flagellar pocket  
99 neck region, where the FAZ in *Leishmania* is found [3]. Despite the conservation in protein content  
100 there are two major organisational differences between the cytoskeletal elements of the *Leishmania*  
101 and *T. brucei* FAZ [3]. Firstly, in *Leishmania* the microtubule quartet and FAZ filament are not found  
102 beneath the primary region of flagellum-to-cell body attachment but are positioned approximately a  
103 quarter turn anti-clockwise from this attachment region, in cross sections through the FAZ area looking  
104 towards the posterior of the cell [3]; however, near the anterior cell tip the attachment region  
105 broadens and here the distal end of the microtubule quartet and FAZ filament connect into the  
106 attachment zone proper. Secondly, in *Leishmania* the fibres of the FAZ within the flagellum do not  
107 connect with the PFR but instead connect the flagellum membrane with the axonemal microtubule  
108 doublets [3]. Despite the physical separation between the cytoplasmic components of the FAZ and the  
109 primary attachment region in *Leishmania*, there is still a clear connection between the flagellum and  
110 the cell body membranes in this region, and the membrane attachment area is linked to the flagellar  
111 cytoskeleton.

112 In *T. brucei* the FAZ has additional functions beyond maintaining lateral flagellum attachment to the  
113 cell body, including a key role in cytokinesis and cell morphogenesis. During the cell cycle a new  
114 flagellum and associated FAZ are assembled alongside the existing flagellum and the distal end of the  
115 FAZ is the site for cytokinesis furrow ingression [16–18]. Moreover, the distal end of the growing FAZ  
116 is associated with a complex of proteins that are important for cytokinesis [19–24] and, the depletion  
117 of flagellum FAZ domain proteins such as ClpGM6 and FLAM3, reduced FAZ length, resulting in the  
118 misplacement of the cleavage furrow and the production of shorter cells [25,26].

119 We have recently begun to investigate the function of the FAZ in *Leishmania* and have interrogated  
120 the function of FAZ5, a protein with multiple transmembrane domains that is likely a component of

121 the primary attachment region in the intermembrane FAZ domain [27]. The deletion of FAZ5 caused  
122 the loss of flagellum attachment to the cell body along the flagellar pocket neck region but this did not  
123 affect the growth of these cells in culture and there were no obvious cytokinesis defects. This contrasts  
124 to *T. brucei*, where flagellum detachment by depletion of FAZ proteins such as FLA1 (intermembrane  
125 domain), FAZ2 and CC2D (cell body domain) caused cytokinesis defects and cell death [9,11,13].  
126 However, the FAZ5 mutant cells were shorter and wider, with an altered flagellar pocket architecture,  
127 indicating that the FAZ in *Leishmania* has a role in cell morphogenesis [27].

128 The separation of the primary attachment region from the FAZ filament and microtubule quartet in  
129 *Leishmania* provided an opportunity to interrogate the function of these parts in isolation from the  
130 other FAZ domains [3]. Here, we focussed on the function of FAZ2 in *Leishmania* as in *T. brucei* this  
131 protein was identified as an essential FAZ filament component [13]. Deletion of FAZ2 in *Leishmania*  
132 was not lethal; however, many cells displayed a distinct phenotype: cells post-division were connected  
133 to each other by a membranous bridge structure between their flagella. Thus, although not a  
134 prominent feature of *Leishmania* the phenotype described here reveals an important role for the FAZ  
135 filament in cell morphogenesis.

136

## 137 **Results**

### 138 **Deletion of FAZ2 impaired cell segregation after cytokinesis**

139 To understand the role of the FAZ filament in *Leishmania*, we investigated the function of FAZ2  
140 (LmxM.12.1120), which was identified as an essential FAZ filament protein in *T. brucei* [13]. We  
141 generated a FAZ2 null mutant in *Leishmania mexicana* by sequential replacement of FAZ2 genes with  
142 antibiotic resistance markers. The integration of the resistance markers and the loss of the FAZ2 open  
143 reading frame were confirmed by PCR (S1A Fig). The parental cell line in which the deletions were  
144 performed expressed SMP1 endogenously tagged at its C-terminus with eGFP-Ty [27]. SMP1 is an



145 integral flagellum membrane protein and the tagged version acts as a marker for the flagellum  
146 membrane, enabling rapid analysis of changes to the flagellar pocket region of the *Leishmania* cell.  
147 We were readily able to generate the FAZ2 null mutant and the overall cell morphology was unaffected  
148 by the loss of FAZ2 (Fig 1B). However, the null mutant grew at a slower rate than the parental cell line  
149 with a slightly longer average doubling time in comparison the parental cells (6.8 ( $\pm$ 0.2) hours vs 6.0  
150 ( $\pm$ 0.1) hours, t-test  $p=0.002$ ) (Fig 1C). In the culture, we noticed the presence of cell 'rosettes' and cells  
151 connected to each other via their flagella (Fig 1D). To quantify this phenomenon, we analysed the cells  
152 directly from culture (Fig 1E). In the FAZ2 null mutant there was an increase in the percentage of cell  
153 rosettes (0.7% to 7%) and cells connected via their flagella (0% to 23.8%), with a concomitant drop in  
154 individual cells with one flagellum (73.4% to 58%). This suggests that there was a defect in cell  
155 segregation in the FAZ2 null mutant.

156 In *Leishmania* assembly of a new flagellum, and the duplication and segregation of the kinetoplast  
157 (mitochondrial DNA) and nucleus occur at set points during the cell cycle; therefore, the number of  
158 flagella, kinetoplasts and nuclei in a cell can be used to define its cell cycle stage. To assess whether  
159 there was a defect in cytokinesis, we categorised the cells based on their number of flagella,  
160 kinetoplasts and nuclei (Fig 1F). FAZ2 null mutant populations had a reduced number of 1K1N1F cells,  
161 with the appearance of cells connected via their flagella. The drop in 1K1N1F cells which was matched  
162 by an increase in cells connected by their flagella suggests that cytokinesis was unaffected but there  
163 was a problem with cell segregation, which manifested in the phenotype of the unique flagellum-to-  
164 flagellum connection.

165 We saw many instances of two cells that were connected via their flagella with the connection point  
166 a short distance from the anterior tip of the cell body (Fig 1G, S1 Movie, S2 Movie). When the flagella  
167 of connected cells were lying side-by-side at the base, a bridge was observed connecting the two  
168 flagella. In addition, on the flagellum of  $\sim$ 14% of 1F cells ( $n=65$ ) a small SMP1 structure near the  
169 flagellum base was seen; this structure was not observed on 1F parental cells ( $n=70$ ) (Fig 1G). Given

170 its location and presence only in FAZ2 null mutant cells, this SMP1-positive structure was likely to  
171 represent a remnant of the bridge that had previously connected two cells via their flagella.

172 To ensure that this flagellum-to-flagellum connection was a specific consequence of the loss of FAZ2  
173 we introduced an ectopic copy of FAZ2 tagged with Ty-mChFP using an endogenous expression  
174 plasmid [27]. Expression of the Ty-mChFP::FAZ2 'add-back' protein was confirmed by western blotting,  
175 and the add-back protein localised to the expected position in the flagellar pocket neck by  
176 fluorescence microscopy (Fig 1B, S1B Fig). The growth rate of the FAZ2 add-back cell line was similar  
177 to that of the parental cells and no cells connected by their flagella were observed, indicating the  
178 effects described above were indeed due to the loss of FAZ2 (Fig 1C, E, F). To determine the  
179 reproducibility of the flagellum-to-flagellum connections we generated three further FAZ2 null  
180 mutants using the CRISPR/Cas9 competent cell line C9/T7 [28], with deletion of FAZ2 confirmed by  
181 PCR (S2A Fig). We then counted the different cell types present in the culture and the cell cycle stage  
182 of these different clones (S2B-C Fig). Both rosettes and cells connected via their flagella were readily  
183 observed confirming the reproducibility of this phenotype.

#### 184 **Cells lacking FAZ2 have a shorter flagellar pocket and a very short FAZ filament**

185 Our previous work has shown that the deletion of FAZ5 led to changes in flagellar pocket shape [27].  
186 To determine if there was a change in the morphology of the flagellar pocket after FAZ2 deletion we  
187 measured the distance between the kinetoplast and the anterior cell tip (an estimate for flagellar  
188 pocket length) (Fig 2A, S2D Fig). This distance was reduced in the FAZ2 null mutant in comparison with  
189 the parental and the FAZ2 add-back cells, suggesting that a shorter length of flagellum is housed within  
190 a flagellar pocket. To investigate the morphological changes to the flagellar pocket in greater detail  
191 we examined the anterior end of the cell by thin-section transmission electron microscopy (TEM) (Fig  
192 2B, D). Thin-section TEM revealed that the overall organisation of the flagellar pocket was maintained,  
193 with both the bulbous region and the flagellar pocket neck region present. However, longitudinal  
194 images of the flagellar pocket revealed that both the length of the bulbous region (i.e. the distance

195 between the basal body – i), and the length of the neck region (i.e. the distance between the flagellar  
196 pocket collar and between the flagellar pocket collar and the flagellum exit point – ii) were reduced in  
197 the null mutant (Fig 2B, C). This correlates with the reduced length of the flagellar pocket observed by  
198 light microscopy (Fig 2A).

199 **Figure 2.** FAZ2 null mutants had an altered flagellar pocket shape and reduced level of flagellum  
200 attachment. (A) Representative light microscopy image of a parental cell with Hoechst stained DNA  
201 (cyan), with kinetoplast to anterior cell tip measurement indicated. Measurement of the distance  
202 between the kinetoplast and the anterior end of the cell body for the parental, FAZ2 null mutant and  
203 FAZ2 add back cells. These measurements were done independently 3 times on at least 100 1K1N  
204 cells. The mean of each replicate is plotted as a circle with the mean and s.d. of these individual means  
205 plotted as black lines. An unpaired, two tailed t-test was used to calculate the p value. (B)  
206 Representative electron micrograph of longitudinal section through the flagellar pocket of a parental  
207 cell and FAZ2 null mutant cell. (i) represents the distance between the basal body and the flagellar  
208 pocket collar and (ii) represents the distance between the flagellar pocket collar and the anterior cell  
209 tip. Scale bar is 500 nm. (C) Measurement of i and ii highlighted in (B). Each measurement (parental  
210 n=34, FAZ2 null mutant n=35) from one biological replicate was plotted with the mean represented as  
211 a red line. (D) Quantitation of the different cross sectional profiles across the flagellum and cell body  
212 observed as the flagellum extends through the flagellar pocket into the flagellar pocket neck region  
213 between the parental cells (n=39) and FAZ2 null mutant (n=58) from one biological replicate. Electron  
214 micrographs illustrate the two profiles based on the presence or absence of the attachment between  
215 the flagellum and the cell body. Scale bar is 200 nm.

216 The *T. brucei* FAZ2 homolog has a role in maintaining the attachment of the flagellum to the cell body  
217 [13]. To examine if FAZ2 has a similar function in *Leishmania*, random TEM cross sections through the  
218 flagellar pocket were scored for the presence or absence of flagellum attachment to the cell body in  
219 both the parental and null mutant cells (Fig 2D). Deletion of FAZ2 led to a reduction in the number of

220 cross sections in which attachment was observed; however, flagellum attachment to the cell body was  
221 still seen in nearly half the images examined. Next, we investigated whether FAZ2 deletion had overall  
222 effects on cell morphogenesis, by measuring the cell body length and width, and the flagellum length,  
223 in 1F1K1N cells of for parental, FAZ2 null mutant and FAZ2 add-back populations (S1C-E Fig). There  
224 were only minimal differences in these parameters between the different cell lines, showing that loss  
225 of FAZ2 did not have a great impact on overall cell morphogenesis.

226 To complement the thin-section TEM imaging we examined the flagellar pocket of the FAZ2 null  
227 mutant by serial electron tomography (Fig 3, S3 Movie, S4 Movie), which allows detailed analysis of  
228 the FAZ cytoskeletal elements [3]. Tomography data confirmed that the thin-section TEM images  
229 showing that the broad features of flagellar pocket organisation - the bulbous lumen and a flagellar  
230 pocket neck region demarcated by the collar were present in the null mutant (Fig 3D). Moreover, the  
231 microtubule quartet was observed in its normal position wrapping around the bulbous lumen of the  
232 flagellar pocket, passing through a gap in the flagellar pocket collar and extending into the flagellar  
233 pocket neck region (Fig 3D). However, the FAZ filament marked by the white asterisk, which is  
234 normally found adjacent to the microtubule quartet in the flagellar pocket neck region, was much  
235 reduced in the FAZ2 null mutant, forming a short stub near the flagellar pocket collar (Fig 3D, G). In  
236 the parental cell the flagellum is connected to the cell body through a regular series of electron dense  
237 junctional complexes that form the primary attachment region (Fig 3A, B). In the equivalent position  
238 in the FAZ2 null mutant there were regions where the flagellar membrane appeared attached to the  
239 cell membrane, but these were not associated with the electron dense junctional complexes in the  
240 cell body (Fig 3D-F). An important feature of the *Leishmania* anterior cell tip is its asymmetry with the  
241 side associated with flagellum attachment extending a short distance further out along the flagellum  
242 than the surrounding cell body (Fig 3B). Interestingly, in the FAZ2 null mutant tomogram there was a  
243 'finger' of cell body that extended along the flagellum, partially wrapping around the flagellum (Fig  
244 3D, H, I). This extension did not contain cytoplasmic (sub-pellicular) microtubules, but contained  
245 electron dense structures reminiscent of the junctional complexes, associated with a region of

246 attachment between the cell body and flagellum membrane. However, these densities were not  
247 arranged in the orderly pattern observed in the parental cell (Fig 3D, H, I).

248 **Figure 3.** FAZ2 null mutants had a shorter FAZ filament and an extension of the anterior cell tip along  
249 the flagellum. (A) Model of flagellar pocket generated from tomogram of parental cell. The cell body  
250 membrane is blue with the flagellum membrane in grey. The microtubule quartet (red) is nucleated  
251 close to the basal bodies, then wraps around the flagellar pocket bulbous domain before extending  
252 along the flagellar pocket neck. Additional microtubules (red) extend away from the flagellar pocket  
253 into the cell on the opposite side to the microtubule quartet. FAZ filament in yellow marked by a white  
254 asterisk runs alongside the microtubule quartet in the flagellar pocket neck. Small orange spheres  
255 mark the junctional complexes attaching the flagellum to the cell body. (B) Longitudinal tomogram  
256 slice through model in (A) along dotted black line. The regular electron dense junctional complexes  
257 mediate attachment of the flagellum (white bracket). The attached side of the cell body extends  
258 further along the flagellum than the opposite side. (C) Cross-sectional tomogram slice through model  
259 in (A) along dotted black line. White asterisk marks the FAZ filament. (D) Model of flagellar pocket  
260 generated from tomogram of FAZ2 null mutant. The cell body membrane is blue with the flagellum  
261 membrane in grey. The FAZ filament in yellow is much shorter (white asterisk), whilst the microtubule  
262 quartet appeared unaffected by FAZ2 deletion. A projection of cell body extended along the flagellum.  
263 There were fewer junctional complexes (small orange spheres) with the majority found in the cell body  
264 projection (black arrowheads). (E) Longitudinal slice through model in (D) along the dotted black line.  
265 The flagellum was still connected to the cell body (white bracket) and distinct fibres connecting the  
266 cell body and flagellum membrane were seen (white arrowheads). (F) Cross-sectional slice through  
267 model in (D) along the dotted black line. (G) Enlarged image of area indicated by a dotted box in (D)  
268 showing the shorter FAZ filament (white asterisk). (H) Enlarged image of area indicated by a dotted  
269 box in (D). The cell body membrane is blue with the flagellum membrane in grey and the sub-pellicular  
270 microtubules are in red. Junctional complexes are represented by orange spheres. (I) Longitudinal slice

271 through (H) showing the disorganised junctional complexes in the cell body extension (white arrows)  
272 with the sub-pellicular microtubules marked by the white arrowhead. Scale bar is 200 nm.

273

#### 274 **Deletion of FAZ2 disrupts the molecular organisation of the FAZ**

275 The loss of FAZ2 resulted in errors in the morphogenesis of the anterior cell tip that appeared to affect  
276 the organisation and position of the FAZ. We therefore wanted to analyse the changes to the  
277 molecular structure of the major FAZ domains: i) the cell body, ii) the intermembrane and iii) the  
278 flagellum in more detail [5]. We endogenously tagged FAZ proteins that represent these different  
279 domains with mChFP in the parental and FAZ2 null mutant cells and then imaged them with  
280 fluorescence microscopy (Fig 4). FAZ1 is found within the cell body FAZ domain, FAZ5 is located on the  
281 cell body side of the intermembrane domain, whereas FLA1BP is on the flagellum side of the  
282 intermembrane domain and ClpGM6 localises to the flagellum domain [3,10,12,25]. In addition, FAZ10  
283 was tagged as a marker of the flagellum exit point [3]. There was no change in the localisation of FAZ10  
284 between the parental and FAZ2 null mutant cells, with the FAZ10 signal forming a ring around the  
285 flagellum exit point from the cell body in both cell lines (Fig 4A). In the parental cells, the FAZ1 signal  
286 had the expected pattern with a ring around the flagellum and a short line parallel to the flagellum  
287 within the flagellar pocket neck; however, in the FAZ2 null mutant only the ring structure was  
288 observed, which was more pronounced than in the parental cells (Fig 4B). The loss of the short FAZ1  
289 line signal correlates with the reduction in the length of the FAZ filament observed by cellular electron  
290 tomography.

291 **Figure 4.** Deletion of FAZ2 affected the localisation of FAZ proteins in the FAZ membrane and flagellum  
292 domains but had little effect on FAZ proteins in the cell body domain. (A-E) Images of cells expressing  
293 FAZ proteins tagged with mChFP (magenta) representing the different FAZ domains in parental and  
294 FAZ2 null mutant cells. The flagellum membrane protein SMP1 is tagged with eGFP (green) and the  
295 DNA is stained with Hoechst 33342 (cyan). The inset shows an enlarged image of the FAZ protein

296 localisation. Scale bar, 5  $\mu$ m. F) Schematic of the FAZ domain organisation and anterior cell tip  
297 structure in the parental and FAZ2 null mutant cells.

298 FAZ5 signal was apparent as a short line parallel to the flagellum within the flagellar pocket neck region  
299 of parental cells, but in the FAZ2 null mutant the FAZ5 signal was not in the expected position; it  
300 appeared as a short line of signal alongside the proximal part of the flagellum, extending beyond the  
301 anterior cell tip (Fig 4C). This signal beyond the cell tip correlates with the extension of the cell body  
302 observed by electron microscopy (Fig 3D, H, I). FLA1BP and ClpGM6 had a similar localisation pattern  
303 to each other. In parental cells the FLA1BP and ClpGM6 signal appeared as a short line within the  
304 flagellum in the flagellar pocket neck region, which was asymmetrically positioned to one side of the  
305 flagellum (Fig 4D, E). However, in the FAZ2 null mutant cells FLA1BP and ClpGM6 were localised to a  
306 short region on one side of the flagellum with the strongest signal observed beyond the end of the  
307 cell body (Fig 4D, E). Together these data show that loss of FAZ2 resulted in changes to the organisation  
308 of the FAZ with the intermembrane and flagellum FAZ domains (FAZ5, FLA1BP, ClpGM6) disconnected  
309 from the cell body FAZ domain (FAZ1) and now localised outside the FAZ area in the neck region of  
310 the flagellar pocket, and potentially associated within an extension of the anterior cell tip found in the  
311 FAZ2 null mutant (Fig 4F). The mislocalisation of FAZ proteins in the FAZ2 null mutant provides  
312 evidence for a definitive role for the FAZ in anterior cell morphogenesis.

### 313 **The flagellum-to flagellum connection in the FAZ2 null mutant are mediated by FAZ proteins**

314 Next, we wanted to examine the structure of the flagellum-to-flagellum connection in detail. To do  
315 this we examined the localisation of the endogenously tagged FAZ proteins in cells that were  
316 connected via their flagella (Fig 5). In connected FAZ2 null mutant cells the localisation of FAZ10 and  
317 FAZ1 matched that observed in cells with unconnected flagella (Fig 5A, B). In cells expressing FAZ5  
318 with their flagella connected, the most intense FAZ5 signal coincided with the connection and fainter  
319 signals were observed extending from the connection along the flagellum towards the cell body (Fig  
320 5C). FLA1BP had a similar localisation to that of FAZ5, with the strongest signal coinciding with the

321 connection between the flagella and fainter signals extending from this point along the flagella  
322 towards the cell bodies (Fig 5D). In cells with connected flagella ClpGM6 signal was present along the  
323 flagellum for ~2  $\mu\text{m}$  from the anterior cell tip to the connection region. Unlike FAZ5 and FLA1BP, the  
324 ClpGM6 signal did not coincide with the structure mediating attachment between the two flagella and  
325 instead appeared within the flagellum directly adjacent to the connection (Fig 5E). This shows that the  
326 connection between the flagellum was associated with FAZ proteins from the intermembrane and  
327 flagellum FAZ domains.

328 **Figure 5.** Flagella-to-flagella connections contained FAZ membrane domain proteins. (A-E) Images of  
329 FAZ2 null mutant cells connected via their flagella expressing FAZ proteins tagged with mChFP  
330 (magenta) representing the different FAZ domains. The flagellum membrane protein SMP1 is tagged  
331 with eGFP (green) and the DNA is stained with Hoechst 33342 (cyan). The enlarged image shows the  
332 flagella-to-flagella connection in detail, with FAZ5 and FLA1BP clearly overlapping with the  
333 connection. Scale bar, 5  $\mu\text{m}$ .

334 We used scanning electron microscopy (SEM) to examine the flagellum-to-flagellum connection in the  
335 FAZ2 null mutant to determine at which point during the cell cycle it appeared (Fig 6A, B). The SEM  
336 micrographs showed that the two flagella were connected when the new flagellum was very short and  
337 had only just emerged from the flagellar pocket neck (Fig 6A, B). The connection persisted throughout  
338 the remainder of the cell cycle and for the majority of this time the flagellum-to-flagellum connection  
339 was not associated with the cell body. To examine the flagellum-to-flagellum connection further we  
340 used high-resolution SEM (Fig 6C) and serial electron tomography to generate a 3D reconstruction (Fig  
341 6D, E, S5 Movie). The high-resolution SEM micrographs showed that the connection between the  
342 flagella was not direct and was instead mediated by an additional membrane structure that acted as  
343 an intermediate. The electron tomography showed that in the intermediate connecting structure  
344 there were electron dense regions underlying the membrane that connected to each flagellum, which  
345 were associated with connecting fibres inside the flagella. These electron dense structures were highly



346 reminiscent of the FAZ junctional complexes and correlate with the FAZ protein localisation,  
347 suggesting that the flagellum-to-flagellum connections between FAZ2 null mutant cells are  
348 orchestrated by FAZ proteins.

349 **Figure 6.** Flagella-to-flagella connections were mediated by a small membrane bound bridge  
350 structure. A, B) Conventional scanning electron micrographs of parental (A) and FAZ2 null mutant (B)  
351 cells through the cell cycle. The connection is formed between the flagella (white arrow) as soon as  
352 the new flagellum exits the cell body. Scale bar is 5  $\mu$ m. C) High-resolution scanning electron  
353 micrographs showing the bridge that mediates flagellum-to-flagellum connection (the white box  
354 indicates the position of the area enlarged beneath). Scale bar, 5  $\mu$ m (A, B, C image on the left) and  
355 500 nm (C, lower panel). D) Slices through the tomogram showing the connections (white arrow)  
356 between the flagellum and the connecting bridge structure. The asterisks indicate the electron  
357 density within the connecting structure. Scale bar, 200 nm. E) Model of the connection generated  
358 from tomogram of connected flagella in the FAZ2 null mutant.

### 359 **FAZ2 null mutant is unable to develop and proliferate in the sand fly vector**

360 Given that the FAZ2 null mutant grew in culture we investigated whether the defect in anterior cell  
361 membrane resolution and the flagellum-to-flagellum connections affected the ability of the parasite  
362 to progress through its life cycle. We fed sand flies with blood containing either the parental, null  
363 mutant or add back cell line. We examined the development of the parasite by dissecting sand flies  
364 either 1-2 days or 6-8 days after the blood meal and categorised the parasite burden and its location  
365 in the sand fly gut (Fig 7, S3 Fig). All three cell lines were able to establish an infection in the midgut  
366 1-2 days after the blood meal, but the parasite burden in the null mutant was considerably lower than  
367 in the parental and add-back cell lines (Fig 7A). At 6-8 days after the blood meal very few sand flies  
368 were infected with the null mutant whereas for both the parental and add-back cells, the infection  
369 rate was above 70%, with more than 60% of sand flies having a heavy parasite burden (>1000  
370 parasites/fly; Fig 7A). In the few sand flies in which the FAZ2 null mutant was present they were

371 restricted to the midgut and had not colonised the stomodeal valve unlike the parental and add-back  
372 cells (S3 Fig). Together this showed that the deletion of FAZ2 decreased dramatically the proliferation  
373 and development of the parasite in the sand fly, suggesting that the defect in late stage resolution of  
374 cell segregation has stronger implications in the environment of the sand fly gut compared with the  
375 conditions of the in vitro culture. A potential alternative explanation for the inability of the FAZ2 null  
376 mutant to proliferate and develop in the sand fly is that the cells had a defect in motility (effective  
377 displacement) due to the flagellum-to-flagellum connection. To explore this idea, we examined the  
378 motility of the parental, null mutant and add-back cells by tracking the movement of 1000s of cells  
379 (S4A Fig). The cell tracks showed a clear reduction in processive movement in the FAZ2 null mutant in  
380 comparison with the parental and FAZ2 add-back cells. We then plotted the mean cell speed and the  
381 directional persistence of these cells (S4B-C Fig), which showed a reduction in directional persistence  
382 of the FAZ2 null mutant. To examine this further we manually extracted the tracks of 50 1F cells for  
383 the parental, null mutant and add back cells and 50 F-to-F cells for the null mutant. For the parental  
384 and add back cells the 50 1F cell tracks had a similar distribution of mean cell speed and directional  
385 persistence, which matched that of their respective overall cell tracks. For both the null mutant 50 1F  
386 and F-to-F cell tracks there was reduced directional persistence in comparison to the parental cells,  
387 with a greater reduction in directional persistence seen for the F-to-F tracks. This suggests that the  
388 deletion of FAZ2 reduces the ability of the parasite to move in a directional manner whether there is  
389 a flagellum-to-flagellum connection or not. However, the flagellum-to-flagellum connection does not  
390 always cause an impediment to directional movement.

391 **Figure 7.** FAZ2 deletion severely affected the ability of *Leishmania* to proliferate and develop in the  
392 sand fly and dramatically reduced pathogenicity in the mouse. A) Analysis of sand fly infections using  
393 parental, FAZ2 null mutant and FAZ2 add-back cells. After 1-2 days post blood meal and 6-8 days post  
394 blood meal sand flies were dissected (number indicated above the column) and the parasite load in  
395 each fly was measured as heavy (1000+ parasites), moderate (100-1000 parasites), weak (1-100  
396 parasites). This is the combined data from two independent sand fly infection experiments. B)

397 Representative electron micrographs of longitudinal section through the flagellar pocket of a parental  
398 cell and FAZ2 null mutant axenic amastigotes. (i) represents flagellar pocket length, (ii) represents  
399 flagellum length, (iii) represents width of the flagellum at the constriction point. Scale bar is 1000 nm.  
400 C) Graphs for the 3 measurements in (B) with the mean plotted and error bars showing standard  
401 deviation for parental and FAZ2 null mutant axenic amastigotes. For flagellum length – parental cells  
402 n=16, FAZ2 null mutant n=24; for flagellar pocket length – parental cells n=16, FAZ2 null mutant n=27;  
403 for flagellum width – parental cells n=18, FAZ2 null mutant n=29. D) Measurement of mouse footpad  
404 lesion size during an 8-week infection time course with parental, FAZ2 null mutant and FAZ2 add-back  
405 cells. Error bars indicate standard deviation. A two-tailed unpaired t-test was used for the pairwise  
406 comparisons of the parental versus FAZ2 null mutant and FAZ2 null mutant versus FAZ2 add back. E)  
407 Measurement of parasite burden at the end of the 8-week infection time course in the footpad lesion  
408 and the lymph node for the parental, FAZ2 null mutant and FAZ2 add back cells. Parasite number from  
409 each infection is plotted with the mean and the 95% SEM interval indicated, and p values were  
410 calculated using a two-tailed unpaired Student's t-test (n=5).

#### 411 **FAZ2 deletion affects amastigote structure and reduces pathogenicity in the mouse**

412 In the mammalian host, *Leishmania* is an intracellular parasite that resides within a parasitophorous  
413 vacuole and has an amastigote morphology with a much shorter flagellum that only just extends  
414 beyond the cell body [29]. During the promastigote to amastigote differentiation there is a  
415 reorganisation of the FAZ structure and changes in FAZ protein localisation [3]. To understand whether  
416 FAZ2 deletion affected amastigote formation we triggered differentiation of promastigotes in vitro by  
417 reducing the pH and increasing the temperature [30]. Over 72 hours the FAZ2 null mutant  
418 differentiated successfully and there were no apparent differences between the morphology of the  
419 null mutant amastigote and that of the parental and add-back amastigotes (S5A Fig).

420 To investigate the flagellar pocket region of amastigotes in greater detail we imaged these cells by  
421 thin-section TEM (Fig 7B). The null mutant flagellar pocket still had both the bulbous lumen and neck

422 region. In longitudinal sections the flagellum appeared to extend beyond the cell body farther (Fig 7B).  
423 Morphological analysis revealed that the flagellum of the FAZ2 null mutant amastigotes was on  
424 average slightly longer than that of parental amastigotes ( $2460\pm 440$  nm (n=24) vs  $2194\pm 252$  nm  
425 (n=16)), with the mean length of the flagellar pocket essentially unchanged; however, the variations  
426 in flagellar/flagellar pocket length in the mutant cells was much greater (Fig 7C). Moreover, we noticed  
427 that the constriction point at the distal end of the flagellar pocket neck was wider in the FAZ2 null  
428 mutant than the parental cells (Fig 7C,  $252\pm 38$  nm (n=29) vs  $195\pm 57$  nm (n=18)). This showed that  
429 deletion of FAZ2 affected flagellar pocket morphogenesis in the amastigote form.

430 Even though the FAZ2 null mutant was able to differentiate in vitro, we wanted to test its potential  
431 for growth in a mammalian host. We infected the footpads of mice with the parental, null mutant and  
432 add-back parasites and examined the infection progress over an 8-week time course (Fig 7D). Initially,  
433 infection with the FAZ2 null mutant resulted in an increase in footpad swelling over the first two weeks  
434 after infection, but beyond this point there was no further swelling, unlike that observed in parental  
435 and add-back infections. At the end of the time course the null mutant had caused much less swelling  
436 than the parental cells, with the add-back cells causing an intermediate level of swelling. Next, we  
437 assessed the parasite burdens in the footpad and lymph nodes (Fig 7E). There was a significantly  
438 reduced parasite burden in both the footpad and lymph nodes of those mice infected with the null  
439 mutant in comparison to the parental cells with the FAZ2 add back restoring the parasite numbers.  
440 The loss of FAZ2 had a great effect on the ability of the parasite to cause disease in the mouse,  
441 contrasting with the subtle phenotype observed in vitro.

442 A potential explanation for the reduced pathogenicity of the FAZ2 null mutant was that these cells  
443 were unable to establish an infection in phagocytic cells. To test this idea, we infected bone marrow  
444 derived macrophages with parental, null mutant and add-back cells for 2 hours and then followed the  
445 infection over 72 hours using stationary phase promastigotes (S5B-D Fig). The FAZ2 null mutant cells  
446 reached stationary phase with the same kinetics as the parental and add-back cells, though they had

447 a lower final cell density. The infection indices (number of infected cells, number of parasites/cell)  
448 were similar between all the cell lines, indicating that the null mutant was still able to infect  
449 macrophages in vitro effectively (S5C-D Fig).

450

## 451 **Discussion**

452 Accurate and successful cell division in *Leishmania* involves a new flagellum elongating alongside the  
453 old flagellum with the two flagella initially occupying the same flagellar pocket [31,32]. The flagellar  
454 pocket then divides to generate two separate flagellar pockets each with its own flagellum. The cell  
455 membrane then ‘folds in’ forming a cytokinesis furrow that proceeds from anterior to posterior  
456 generating two daughter cells [31]. The FAZ2 null mutant had a slightly reduced growth rate, which  
457 was associated with defects in the morphogenesis of the anterior cell tip. This demonstrates an  
458 important role for FAZ2 and the FAZ filament in the determination and maintenance of anterior cell  
459 tip morphology.

460 In parental cells the anterior cell tip around the flagellum exit site is asymmetric, with the side  
461 associated with the FAZ extending slightly further alongside the flagellum [3,33]. We now demonstrate  
462 that this asymmetry is set by the FAZ, because deletion of FAZ2 causes an extension of the anterior  
463 cell tip, whilst deletion of FAZ5 results in the loss of the anterior cell tip asymmetry (Fig 3, [27]). In  
464 parental cells, the cell body FAZ domain and the intermembrane and flagellum FAZ domains are found  
465 in the same region along the length of the flagellum [3]; however, in the FAZ2 null mutant these  
466 domains did not coincide, with the intermembrane and flagellum FAZ domain proteins being outside  
467 the FAZ region, distally separated from the FAZ filament remnant in the cell body. The exaggerated  
468 asymmetric extension containing intermembrane and flagellum FAZ domain components does not  
469 contain sub-pellicular microtubules, suggesting that it is ‘independent’ from the cell body proper. The  
470 simplest explanation for this phenotype is that it, in the absence of FAZ2, intermembrane and  
471 flagellum FAZ assemblage is ‘released’ from the physical link to the FAZ filament, and moves along the

472 flagellum, within an anterior cell extension that elongates as the new flagellum extends (Fig 8). Thus,  
473 an important function for FAZ2 is forming the connection between the cell body FAZ domain and the  
474 intermembrane domain.

475 **Figure 8.** Model for the generation of the flagella-to-flagella connections. The parental *Leishmania*  
476 promastigote flagellar pocket is shown from two different angles, giving a side-on and plan view of  
477 the FAZ. In the plan view of the FAZ, all the FAZ domains are aligned one on top of the other: flagellum  
478 domain on top, next the intermembrane domain, then finally the cell body domain. In the parental  
479 cell the new flagellum and FAZ (all domains) assemble alongside the existing flagellum/FAZ structure.  
480 In the FAZ2 null mutant the initial assembly of the new flagellum and new FAZ (all domains) is likely to  
481 occur alongside the remnant of the cell body FAZ domain of the old FAZ in the expected position.  
482 However, without FAZ2 the link between the cell body FAZ domain and the intermembrane and  
483 flagellum FAZ domains is not formed or is weak. Thus as the new flagellum and FAZ continue to  
484 assemble, the flagellum and intermembrane FAZ domains are 'released' from their link to the FAZ cell  
485 body domain and move along with the assembling new flagellum within an anterior cell tip extension.  
486 This anterior cell tip extension on the new flagellum is connected to an existing cell tip extension along  
487 the old flagellum. As the new flagellum continues to grow this structure extends farther until it  
488 eventually separates from the cell body to leave the two flagella connected by a membranous bridge  
489 structure.

490 The membranous bridge structure connecting the two flagella in dividing cells was present as soon as  
491 the new flagellum emerged from the flagellar pocket neck and, at this point, it was still connected to  
492 the cell body. As the new flagellum elongated, the connection separated from the cell body. There is  
493 likely to be a limited length that the anterior cell tip membrane can elongate to before the connection  
494 with the cell body breaks, resulting in the formation of the membranous structure connecting just the  
495 two flagella (Fig 8). This bridge structure was positive for SMP1, a flagellum membrane protein,  
496 suggesting that it originates from the flagellum or at least can receive flagellar membrane

497 components. Within the flagellar pocket a boundary function likely operates to ensure that the  
498 flagellum membrane has a distinct protein complement [34]; the loss of this boundary function could  
499 result in the movement of SMP1 to the connecting structure. However, SMP1 was not enriched on the  
500 cell body membrane so a loss of protein positional control seems unlikely. Our data, however, showed  
501 that this connection was likely formed from an extension of the anterior cell tip. In *T. brucei* transient  
502 interactions between flagella of different cells resulted in the transfer of fluorescent proteins between  
503 them [35]. The close apposition of the flagella and the connecting structure might result in the transfer  
504 of SMP1 by a similar mechanism.

505 In the FAZ2 null mutant the membranous bridge between flagella is isolated from the cell body and,  
506 thus, unaffected by cell division resolution, which generates the unusual phenotype of post-division  
507 cells connected by their flagella. In the absence of FAZ2, the anterior cell tip can divide, the collar can  
508 be segregated into two and two flagellar pockets formed and hence, cytoskeletal morphogenesis  
509 appears normal. However, membrane remodelling (cell body and/or flagellum) is compromised,  
510 leading to alterations of asymmetry and errors in the resolution of the anterior cell tip membrane.  
511 This suggest that, in *Leishmania* promastigotes anterior cell tip morphogenesis is FAZ2 dependent.

512 The depletion of FAZ2 in *T. brucei* by RNAi had a catastrophic effect on the cell with the loss of  
513 flagellum attachment and destabilisation of FAZ proteins [13]. However, in *Leishmania* FAZ2 null  
514 mutants, flagellum attachment was reduced but not lost completely and although there were changes  
515 in FAZ protein localisation no large change in FAZ signal was observed by light microscopy. These  
516 differences are likely explained by the differences in the spatial organisation of the FAZ with the  
517 primary attachment zone in *Leishmania* not being as closely associated with the FAZ filament. This  
518 suggests a nuanced function of the *Leishmania* FAZ, with the FAZ filament playing a greater role in cell  
519 tip morphogenesis than actual flagellum attachment.

520 The FAZ2 null mutant parasites were able to survive within the blood meal surrounded by the  
521 peritrophic matrix in the midgut of the sand fly, albeit with a reduced infection density; however,

522 these parasites were unable to proliferate and develop late-stage infections and hence did not  
523 colonise the cardia (i.e. the most anterior part of the thoracic midgut) and the stomodeal valve. A key  
524 step in the development of the parasite in the sand fly is the escape from the peritrophic matrix and  
525 attachment to the midgut epithelium with a recent study showing that motility is important for the  
526 parasite to complete its life cycle in the sand fly [36–38]. We found that the motility of the FAZ2 null  
527 mutant in vitro was impaired likely due to the abnormalities in cell body-flagellum connections and  
528 the flagellum-to-flagellum connection impeding the ability of these cells to move effectively. This loss  
529 of directional movement could explain the lack of growth and development of this mutant in the sand  
530 fly. However, it is technically difficult to observe individual parasite movement in the sand fly,  
531 something that is common to all such studies and complicates the attribution of specific causal  
532 explanations for mutant behaviour. Here, as in other mutant studies, it is more likely that the reduced  
533 infection in the sand fly midgut is multifactorial, resulting from a combination of different phenomena.

534 The *Leishmania* amastigote flagellar pocket has a two-part structure with the bulbous lumen and the  
535 flagellar pocket neck region as found in the promastigote. However, the shortened amastigote  
536 flagellum only just extends beyond the anterior cell tip, and at the distal end of the flagellar pocket  
537 neck there is a constriction that squeezes tightly around the flagellum, coinciding with the localisation  
538 of FAZ2 [3]. The loss of FAZ2 did not affect the ability of the *Leishmania* parasite to differentiate and  
539 the null mutant retained the two-part flagellar pocket organisation. However, the ultrastructure of  
540 the null mutant axenic amastigote flagellar pocket was more variable, with an increase in the width of  
541 the constriction point at the distal end of the flagellar pocket neck, showing that FAZ2 has an  
542 important role in maintaining amastigote flagellar pocket shape.

543 The FAZ2 null mutants were able to infect macrophages in vitro but had a reduced pathogenicity in  
544 vivo, with both a reduction in footpad swelling and parasite numbers recovered from the footpad and  
545 the lymph node. Thus, FAZ2 null mutant amastigotes struggled to survive and replicate over an  
546 extended period in the mouse. As with the promastigote in vivo phenotype there may be multiple



547 effects leading to this result. However, changes to the amastigote flagellar pocket architecture in the  
548 null mutant may have contributed to the loss of pathogenicity in the mouse, because the increase in  
549 the width of the constriction point might well affect the ability of the parasite to control the exchange  
550 of material between the flagellar pocket and the extracellular milieu. The 'relaxed' constriction at the  
551 flagellar pocket exit might also increase parasite exposure to deleterious factors from the  
552 environment, hence reducing cell viability. The deletion of FAZ5 in *L. mexicana* altered the flagellar  
553 pocket architecture with the loss of the flagellar pocket neck region and these changes were  
554 associated with a large reduction in infectivity in the mouse [27]. Together with our results here this  
555 suggests that flagellar pocket architecture is important for parasite pathogenicity in the mammalian  
556 host.

557 In summary, we have shown that the FAZ filament is critical for the morphogenetic resolution of the  
558 *Leishmania* anterior cell tip. This clearly demonstrates the subtleties of the function of the FAZ not  
559 only as a crucial feature for flagellum attachment but also its role in membrane organisation at the  
560 anterior cell tip. This provides a deeper understanding of membrane-cytoskeletal interactions in the  
561 definition of cell form in this parasite.

562

## 563 **Materials and Methods**

### 564 Ethics statement

565 Experiments involving mice were conducted according to the Animals (Scientific Procedures) Act of  
566 1986, United Kingdom, and had approval from the University of York Animal Welfare and Ethical  
567 Review Body (AWERB) committee.

### 568 Cell culture

569 *L. mexicana* (WHO strain MNYC/BZ/1962/M379) promastigotes were grown at 28°C in M199 medium  
570 with 10% foetal calf serum, 40 mM HEPES-NaOH (pH 7.4), 26 mM NaHCO<sub>3</sub> and 5 µg/ml haemin. Cells

571 were maintained in logarithmic growth. Promastigotes were differentiated to axenic amastigotes by  
572 subculturing into Schneider's Drosophila medium with 20% FCS and 25 mM MES-HCl (pH 5.5) at 34°C  
573 with 5% CO<sub>2</sub>, and grown for 72 h without subculture.

#### 574 Generation of FAZZ deletion constructs, tagging constructs and FAZZ add back construct

575 Deletion constructs were generated using fusion PCR as described [39]. Regions comprising 500 bp of  
576 the 5' UTR and 500 bp of the 3' UTR of the *FAZZ* gene were combined with either the hygromycin  
577 resistance gene or the neomycin resistance gene by PCR to generate the deletion constructs. For  
578 tagging the corresponding ORFs and UTRs were cloned into pLEnTv2-YB plasmid [39]. To produce the  
579 add-back cell line, the *FAZZ* gene was cloned into the XbaI and BamHI restriction sites of the  
580 constitutive expression plasmid described in [27]. For the generation of FAZZ null mutants using  
581 CRISPR/Cas9 mediated genome editing, the C9/T7 cell line was transfected with guide and repair  
582 constructs generated by PCR using primers designed on the LeishGedit website using the G00 primer  
583 and the pTBlast and pTPuro plasmids as templates [28]. Constructs were transfected using a  
584 Nucleofector 2b as described previously [39].

#### 585 Light Microscopy

586 For live cell microscopy, cells were washed three times in PBS, resuspended in PBS with Hoechst 33342  
587 (1 µg/ml) and then 5 µl of cell suspensions were placed on a glass slide. The cells were imaged using  
588 either a Leica DM5500B microscope with 100x objective and Neo 5.5 sCMOS camera or a Zeiss  
589 ImagerZ2 microscope with 63x or 100x objective and Hamamatsu Flash 4 camera. For cell swimming  
590 analysis, a 61 s video of 512 frames under darkfield illumination was captured using a 10x objective.  
591 Particle tracks were traced and quantified (mean speed and cell directionality (ratio of velocity to  
592 speed) automatically as previously described [40]. Individual tracks were extracted after manual  
593 inspection for presence of 1 flagellum or cells connected via their flagella, with the track statistics then  
594 plotted as for the entire population.

595

596 Transmission electron microscopy (TEM)

597 Cells were fixed in culture by the addition of glutaraldehyde for a final concentration of 2.5%. After 3  
598 minutes, the cells were centrifuged (at 800g, for 5 min), washed in buffered fixative solution (0.1 M  
599 PIPES-NaOH buffer, pH 7.2, with 2.5% glutaraldehyde and 4% formaldehyde), resuspended in fresh  
600 buffered fixative solution and fixed overnight at 4°C. Cells were then washed five times in 0.1 M PIPES-  
601 NaOH buffer, pH 7.2 (including one 30-min wash in 50 mM glycine in 0.1 M PIPES-NaOH buffer), and  
602 post-fixed in 1% OsO<sub>4</sub> in 0.1 M PIPES-NaOH buffer at 4°C, for 2h. Cells were washed five times in  
603 deionized water, then stained en bloc with 2% aqueous uranyl acetate overnight, at 4°C. Samples were  
604 then dehydrated in ethanol and embedded in Agar 100 resin. Thin-sections were stained with  
605 Reynolds' lead citrate, before imaging on a Tecnai T12, equipped with a OneView 4x4 mega pixel  
606 camera (Gatan).

607 Electron microscopy tomography

608 Ribbons containing serial sections of ~150 nm were produced from samples prepared for TEM as  
609 described above. Sections were stained with Reynolds' lead citrate before imaging at 120 kV, on a  
610 Tecnai T12 with a OneView (Gatan) camera. Each individual tomogram was produced from a total of  
611 240 4K x 4K pixel images (120 tilted images each of 0 and 90° axes, with 1° tilting between images)  
612 acquired automatically using SerialEM. Individual tomograms were produced using eTOMO (IMOD  
613 software package), and consecutive tomograms were then joined to produce serial tomogram  
614 volumes, using eTOMO. Tri-dimensional models from serial tomograms were produced by manual  
615 tracing and segmentation of selected structures using 3Dmod (IMOD software package).

616 Scanning electron microscopy

617 Cells were fixed by adding glutaraldehyde to final concentration of 2.5% into the culture. Cells were  
618 harvested by centrifugation at 800 g for 5 minutes, the supernatant was removed and primary fixative

619 was added (2.5% glutaraldehyde in 100 mM sodium phosphate buffer). After two hours, cells were  
620 washed twice in PBS and settled for 5 minutes onto round glass coverslips treated with poly-L-lysine.  
621 Coverslips were washed two times in PBS and the samples were then dehydrated using increasing  
622 concentrations of ethanol (30%, 50%, 70% and 90% v/v in distilled water, followed by three times in  
623 100% ethanol). Samples were then critical point dried. Coverslips were mounted onto SEM stubs using  
624 silver DAG and coated with gold using a sputter coater. Images were taken on a Hitachi S-3400N  
625 scanning electron microscope at 5 kV with a 5.5 mm working distance. For high-resolution SEM,  
626 samples were imaged at 10 kV in a Zeiss Merlin Compact, using an in-lens SE detector and a 3 mm  
627 working distance.

#### 628 Sand fly infections

629 All parasites were cultivated at 23°C in M199 medium with 20% FCS, 1% BME vitamins, 2% sterile urine  
630 and 250 µg/ml amikin. Before infections parasites were washed three times in saline and resuspended  
631 in defibrinated heat-inactivated rabbit blood at  $1 \times 10^6$  promastigotes/ml. *Lutzomyia longipalpis* were  
632 maintained at 26°C and high humidity on 50% sucrose solution and 14 h light/10 h dark. Sand fly  
633 females, 3-5 days old, were fed through a chick skin membrane [41]. Fully-engorged females were  
634 separated and maintained at 26°C with free access to 50% sucrose solution. They were dissected on  
635 days 1-2 and 6-8 post bloodmeal and the guts were checked for localisation and intensity of infection  
636 by light microscopy. Parasite loads were graded as described previously [42]. Each cell line was used  
637 to infect sand flies in two independent experiments.

#### 638 Macrophage infections

639 Bone marrow derived macrophages (BMDMs) were grown in DMEM with 10% FCS and 10 ng/ml M-  
640 CSF at 37°C with 5% CO<sub>2</sub>. BMDMs were grown to confluence and then used to seed wells at  $2.5 \times 10^4$   
641 cells/well. Promastigotes in log growth were split to  $1 \times 10^5$  cells/ml and grown to stationary phase over  
642 5 days. The stationary phase promastigotes were used to infect the BMDMs for 2 h at a MOI of 5. After  
643 washing the cells to remove any free parasites the infected BMDMs were incubated at 34°C with 5%

644 CO<sub>2</sub> in DMEM for 3 days. At each time point BMDMs were fixed with methanol and the stained with  
645 the DRAQ5 and then imaged. Infected BMDMs and *Leishmania* parasites were then counted.

#### 646 Virulence assessment *in vivo* – footpad measurement and limiting dilution assays

647 All procedures were performed under Home Office Licence and in accordance with Institutionally-  
648 approved protocols. Strain virulence was assessed by footpad swelling and parasite burden [43]. For  
649 experimental infections the parasites had previously been passaged through mice, isolated and  
650 transformed into promastigotes before being used. Groups of 5 female BALB/c mice (4-6 weeks) were  
651 infected subcutaneously at the left footpad using  $2.0 \times 10^6$  stationary promastigotes in 40  $\mu$ l of sterile  
652 PBS. Infections were followed weekly by footpad measurement, and animals culled after 8 weeks  
653 using approved Schedule 1 methods prior to removal of footpad lesions and lymph nodes under sterile  
654 conditions. Samples were kept in M199 supplemented with 5  $\mu$ g/ml gentamycin, and footpads  
655 digested with 4 mg/ml collagenase D for 2 h at 37°C. Lymph nodes and digested tissues were  
656 mechanically dissociated and filtered through a 70  $\mu$ m cell strainer. Homogenates were resuspended  
657 in M199 supplemented with 20% FCS and serial dilutions (2-fold) performed in 96-well clear flat-  
658 bottom plates. Each sample dilution was performed in duplicate and distributed in at least three  
659 plates. Sealed-plates were incubated for 7-10 days at 25°C, wells visually analysed for the presence of  
660 parasites, and number of parasites calculated by multiplying by the dilution factors.

661

#### 662 **Acknowledgements**

663 We thank Dr Eva Gluenz (University of Oxford) for the kind gift of the *L. mexicana* SMP1::eGFP cell  
664 line, Dr Jessica Valli (University of Oxford) for help with the macrophage infection assays. This work  
665 was initiated in the lab of Professor Keith Gull (University of Oxford). We would like to especially thank  
666 Keith Gull for his intellectual input through many conversations which helped to guide the  
667 development of this work and manuscript.

668

669

670 **References**

- 671 1. Hoare CA, Wallace FG. Developmental Stages of Trypanosomatid Flagellates: a New  
672 Terminology. *Nature*. 1966 Dec 17;212(5068):1385–6.
- 673 2. Field MC, Carrington M. The trypanosome flagellar pocket. *Nat Rev Microbiol*. 2009  
674 Nov;7(11):775–86.
- 675 3. Wheeler RJ, Sunter JD, Gull K. Flagellar pocket restructuring through the *Leishmania* life cycle  
676 involves a discrete flagellum attachment zone. *J Cell Sci*. 2016 Feb 15;129(4):854–67.
- 677 4. Vickerman K. On the surface coat and flagellar adhesion in trypanosomes. *J Cell Sci*. 1969  
678 Jul;5(1):163–93.
- 679 5. Sunter JD, Gull K. The Flagellum Attachment Zone: “The Cellular Ruler” of Trypanosome  
680 Morphology. *Trends Parasitol*. 2016 Apr;32(4):309–24.
- 681 6. Sherwin T, Gull K. The cell division cycle of *Trypanosoma brucei brucei*: timing of event markers  
682 and cytoskeletal modulations. *Philos Trans R Soc Lond B Biol Sci*. 1989 Jun 12;323(1218):573–  
683 88.
- 684 7. Portman N, Gull K. The paraflagellar rod of kinetoplastid parasites: from structure to  
685 components and function. *Int J Parasitol*. 2010 Feb;40(2):135–48.
- 686 8. Nozaki T, Haynes PA, Cross GA. Characterization of the *Trypanosoma brucei* homologue of a  
687 *Trypanosoma cruzi* flagellum-adhesion glycoprotein. *Mol Biochem Parasitol*. 1996 Nov  
688 25;82(2):245–55.
- 689 9. LaCount DJ, Barrett B, Donelson JE. *Trypanosoma brucei* FLA1 is required for flagellum  
690 attachment and cytokinesis. *J Biol Chem*. 2002 May 17;277(20):17580–8.
- 691 10. Vaughan S, Kohl L, Ngai I, Wheeler RJ, Gull K. A repetitive protein essential for the flagellum  
692 attachment zone filament structure and function in *Trypanosoma brucei*. *Protist*. 2008  
693 Jan;159(1):127–36.
- 694 11. Zhou Q, Liu B, Sun Y, He CY. A coiled-coil- and C2-domain-containing protein is required for FAZ  
695 assembly and cell morphology in *Trypanosoma brucei*. *J Cell Sci*. 2011 Nov 15;124(Pt 22):3848–  
696 58.
- 697 12. Sunter JD, Varga V, Dean S, Gull K. A dynamic coordination of flagellum and cytoplasmic  
698 cytoskeleton assembly specifies cell morphogenesis in trypanosomes. *J Cell Sci*. 2015 Mar 3;
- 699 13. Zhou Q, Hu H, He CY, Li Z. Assembly and maintenance of the flagellum attachment zone  
700 filament in *Trypanosoma brucei*. *J Cell Sci*. 2015 May 13;

- 701 14. Morriswood B, Havlicek K, Demmel L, Yavuz S, Sealey-Cardona M, Vidilaseris K, et al. Novel  
702 bilobe components in *Trypanosoma brucei* identified using proximity-dependent biotinylation.  
703 *Eukaryot Cell*. 2013 Feb;12(2):356–67.
- 704 15. Moreira BP, Fonseca CK, Hammarton TC, Baqui MMA. Giant FAZ10 is required for flagellum  
705 attachment zone stabilization and furrow positioning in *Trypanosoma brucei*. *J Cell Sci*. 2017  
706 15;130(6):1179–93.
- 707 16. Robinson DR, Sherwin T, Ploubidou A, Byard EH, Gull K. Microtubule polarity and dynamics in  
708 the control of organelle positioning, segregation, and cytokinesis in the trypanosome cell cycle.  
709 *J Cell Biol*. 1995 Mar;128(6):1163–72.
- 710 17. Wheeler RJ, Gull K, Sunter JD. Coordination of the Cell Cycle in Trypanosomes. *Annu Rev*  
711 *Microbiol*. 2019 Sep 8;73:133–54.
- 712 18. Wheeler RJ, Scheumann N, Wickstead B, Gull K, Vaughan S. Cytokinesis in *Trypanosoma brucei*  
713 differs between bloodstream and tsetse trypomastigote forms: implications for microtubule-  
714 based morphogenesis and mutant analysis. *Mol Microbiol*. 2013 Dec;90(6):1339–55.
- 715 19. McAllaster MR, Ikeda KN, Lozano-Núñez A, Anrather D, Unterwurzacher V, Gossenreiter T, et  
716 al. Proteomic identification of novel cytoskeletal proteins associated with TbPLK, an essential  
717 regulator of cell morphogenesis in *T. brucei*. *Mol Biol Cell*. 2015 Jul 1;
- 718 20. Zhou Q, Gu J, Lun Z-R, Ayala FJ, Li Z. Two distinct cytokinesis pathways drive trypanosome cell  
719 division initiation from opposite cell ends. *Proc Natl Acad Sci U S A*. 2016 Mar 22;113(12):3287–  
720 92.
- 721 21. Zhou Q, Hu H, Li Z. An EF-hand-containing Protein in *Trypanosoma brucei* Regulates Cytokinesis  
722 Initiation by Maintaining the Stability of the Cytokinesis Initiation Factor CIF1. *J Biol Chem*.  
723 2016 Jul 8;291(28):14395–409.
- 724 22. Kurasawa Y, Hu H, Zhou Q, Li Z. The trypanosome-specific protein CIF3 cooperates with the  
725 CIF1 protein to promote cytokinesis in *Trypanosoma brucei*. *J Biol Chem*. 2018  
726 29;293(26):10275–86.
- 727 23. Zhou Q, An T, Pham KTM, Hu H, Li Z. The CIF1 protein is a master orchestrator of trypanosome  
728 cytokinesis that recruits several cytokinesis regulators to the cytokinesis initiation site. *J Biol*  
729 *Chem*. 2018 19;293(42):16177–92.
- 730 24. Hu H, An T, Kurasawa Y, Zhou Q, Li Z. The trypanosome-specific proteins FPRC and CIF4  
731 regulate cytokinesis initiation by recruiting CIF1 to the cytokinesis initiation site. *J Biol Chem*.  
732 2019 Nov 8;294(45):16672–83.
- 733 25. Hayes P, Varga V, Olego-Fernandez S, Sunter J, Ginger ML, Gull K. Modulation of a cytoskeletal  
734 calpain-like protein induces major transitions in trypanosome morphology. *J Cell Biol*. 2014 Aug  
735 4;206(3):377–84.
- 736 26. Sunter JD, Benz C, Andre J, Whipple S, McKean PG, Gull K, et al. Flagellum attachment zone  
737 protein modulation and regulation of cell shape in *Trypanosoma brucei* life cycle transitions. *J*  
738 *Cell Sci*. 2015 Jul 6;

- 739 27. Sunter JD, Yanase R, Wang Z, Catta-Preta CMC, Moreira-Leite F, Myskova J, et al. *Leishmania*  
740 flagellum attachment zone is critical for flagellar pocket shape, development in the sand fly,  
741 and pathogenicity in the host. *Proc Natl Acad Sci U S A*. 2019 26;116(13):6351–60.
- 742 28. Beneke T, Madden R, Makin L, Valli J, Sunter J, Gluenz E. A CRISPR Cas9 high-throughput  
743 genome editing toolkit for kinetoplastids. *R Soc Open Sci*. 2017 May;4(5):170095.
- 744 29. Gluenz E, Höög JL, Smith AE, Dawe HR, Shaw MK, Gull K. Beyond 9+0: noncanonical axoneme  
745 structures characterize sensory cilia from protists to humans. *FASEB J Off Publ Fed Am Soc Exp*  
746 *Biol*. 2010 Sep;24(9):3117–21.
- 747 30. Bates PA. Complete developmental cycle of *Leishmania mexicana* in axenic culture.  
748 *Parasitology*. 1994 Jan;108 ( Pt 1):1–9.
- 749 31. Wheeler RJ, Gluenz E, Gull K. The cell cycle of *Leishmania*: morphogenetic events and their  
750 implications for parasite biology. *Mol Microbiol*. 2011 Feb;79(3):647–62.
- 751 32. Ambit A, Woods KL, Cull B, Coombs GH, Mottram JC. Morphological events during the cell cycle  
752 of *Leishmania major*. *Eukaryot Cell*. 2011 Nov;10(11):1429–38.
- 753 33. Santrich C, Moore L, Sherwin T, Bastin P, Brokaw C, Gull K, et al. A motility function for the  
754 paraflagellar rod of *Leishmania* parasites revealed by PFR-2 gene knockouts. *Mol Biochem*  
755 *Parasitol*. 1997 Dec 1;90(1):95–109.
- 756 34. Gadelha C, Rothery S, Morphew M, McIntosh JR, Severs NJ, Gull K. Membrane domains and  
757 flagellar pocket boundaries are influenced by the cytoskeleton in African trypanosomes. *Proc*  
758 *Natl Acad Sci U S A*. 2009 Oct 13;106(41):17425–30.
- 759 35. Imhof S, Fragoso C, Hemphill A, von Schubert C, Li D, Legant W, et al. Flagellar membrane  
760 fusion and protein exchange in trypanosomes; a new form of cell-cell communication?  
761 *F1000Research*. 2016;5:682.
- 762 36. Dostálová A, Volf P. *Leishmania* development in sand flies: parasite-vector interactions  
763 overview. *Parasit Vectors*. 2012 Dec 3;5:276.
- 764 37. Pruzinova K, Sadlova J, Seblova V, Homola M, Votypka J, Volf P. Comparison of Bloodmeal  
765 Digestion and the Peritrophic Matrix in Four Sand Fly Species Differing in Susceptibility to  
766 *Leishmania donovani*. *PLoS One*. 2015;10(6):e0128203.
- 767 38. Beneke T, Demay F, Hookway E, Ashman N, Jeffery H, Smith J, et al. Genetic dissection of a  
768 *Leishmania* flagellar proteome demonstrates requirement for directional motility in sand fly  
769 infections. *PLOS Pathog*. 2019 Jun 26;15(6):e1007828.
- 770 39. Dean S, Sunter J, Wheeler RJ, Hodgkinson I, Gluenz E, Gull K. A toolkit enabling efficient, scalable  
771 and reproducible gene tagging in trypanosomatids. *Open Biol*. 2015 Jan;5(1).
- 772 40. Wheeler RJ. Use of chiral cell shape to ensure highly directional swimming in trypanosomes.  
773 *PLoS Comput Biol*. 2017 Jan;13(1):e1005353.
- 774 41. Volf P, Volfova V. Establishment and maintenance of sand fly colonies. *J Vector Ecol J Soc*  
775 *Vector Ecol*. 2011 Mar;36 Suppl 1:S1–9.



776 42. Myskova J, Votypka J, Volf P. Leishmania in sand flies: comparison of quantitative polymerase  
777 chain reaction with other techniques to determine the intensity of infection. J Med Entomol.  
778 2008 Jan;45(1):133–8.

779 43. Castanys-Muñoz E, Brown E, Coombs GH, Mottram JC. Leishmania mexicana metacaspase is a  
780 negative regulator of amastigote proliferation in mammalian cells. Cell Death Dis. 2012  
781 Sep;3(9):e385.

782

783 S1 Fig (A) Confirmation of FAZ2 gene deletion. gDNA from 4 null mutant clones and the parental cells  
784 was analysed by PCR. PCR confirmed that FAZ2 ORF was no longer present in the null mutant clones  
785 (1-3) and that the resistance markers had integrated correctly in clones (1-3). The neomycin resistance  
786 gene had not correctly integrated into clone 4 and this clone was discarded. FAZ2 null mutant clone 1  
787 was used for all subsequent experiments. The lower less distinct band on the gel (\*) is likely be non-  
788 specific amplification of primer dimers. (B) Western blot confirming expression and expected size (174  
789 kDa) of Ty-mChFP::FAZ2 using the BB2 antibody. The SMP1::eGFP-Ty and BB2 cross reacting band  
790 acted as a loading control. (C, D) Measurement of cell body length and width for parental, FAZ2 null  
791 mutant and FAZ2 add back cells. These measurements were done independently 3 times on at least  
792 50 1K1N cells. The mean of each replicate is plotted as a circle with the mean and standard deviation  
793 of these individual means plotted as black lines. (E) Measurement of flagellum length for parental,  
794 FAZ2 null mutant and FAZ2 add back cells. These measurements were done independently 3 times on  
795 at least 100 1K1N cells. The mean of each replicate is plotted as a circle with the mean and standard  
796 deviation of these individual means plotted as black lines.

797

798 S2 Fig (A) Confirmation of FAZ2 gene deletion. gDNA from 4 null mutant clones and the parental cells  
799 was analysed by PCR. (B) Quantitation of cell types seen in culture for C9/T7 and FAZ2 null mutant  
800 clones. This experiment was performed once and for each cell line  $\geq 84$  cells were counted. (C) Cell  
801 cycle category counts for C9/T7 and FAZ2 null mutant clones. F – flagellum, K – kinetoplast, N –  
802 nucleus, F to F – two cells connected via their flagella. This experiment was performed once and for

803 each cell line  $\geq 110$  cells were counted. (D) Measurement of the distance between the kinetoplast  
804 and the anterior end of the cell body for C9/T7 and FAZ2 null mutant clones. This experiment was  
805 performed once, each measurement is a coloured circle with the mean and s.d. plotted as black  
806 lines. For each cell line  $\geq 62$  cells were measured.

807

808 S3 Fig Migration of *Leishmania* in sand fly gut. Location of *Leishmania* parasites within infected sand  
809 flies at 1-2 and 6-8 days post blood meal. Stacked columns indicate the percentage of infected sand  
810 flies with parasites in various locations within the sand fly. FAZ2 null mutant was unable to migrate to  
811 the stomodeal valve. Percentage of infected flies for each cell line is indicated above each column.  
812 This is the combined data from two independent sand fly infection experiments.

813

814 S4 Fig (A) Swimming tracks from videomicroscopy of parental, FAZ2 null mutant and FAZ2 add back  
815 cells. Cells were imaged for 61 seconds with 512 images taken. Scale bar is 50  $\mu\text{m}$ . (B) Histograms of  
816 the mean speed for parental, FAZ2 null mutant and FAZ2 add back cells for all tracks imaged and for  
817 50 1F cells and 50 F to F cells. (C) Histograms of the directional persistence for parental, FAZ2 null  
818 mutant and FAZ2 add back cells for all tracks imaged and for 50 1F cells and 50 F to F cells. The  
819 histograms and tracks are representative of two independent replicates.

820

821 S5 Fig (A) Images of axenic amastigotes of parental, FAZ2 null mutant and FAZ2 add back cells  
822 expressing SMP1::eGFP-Ty. Scale bar is 5  $\mu\text{m}$ . (B) *Leishmania* macrophage infections. Growth curve of  
823 parental, FAZ2 null mutant and FAZ2 add back cells to stationary phase - average of 3 replicates, mean  
824  $\pm$  s.d is plotted. (C, D) Proportion of infected macrophages and the number of *Leishmania* per infected  
825 macrophage at 0, 24, 48, 72 hours post infection - 0 h time point is after 2 hours of infection and

- 826 removal of cells not taken up. For each time point between 487-1074 macrophages were analysed.
- 827 Mean  $\pm$  s.d. for 3 replicates is shown.
- 828 S1 Movie. Movie of two *Leishmania* cells connected by their flagella.
- 829 S2 Movie. Movie of two *Leishmania* cells connected by their flagella.
- 830 S3 Movie. Movie of tomogram through parental flagellar pocket.
- 831 S4 Movie. Movie of tomogram through FAZ2 null mutant flagellar pocket.
- 832 S5 Movie. Movie of tomogram through bridge structure connecting two flagella.
- 833

# Figure 1

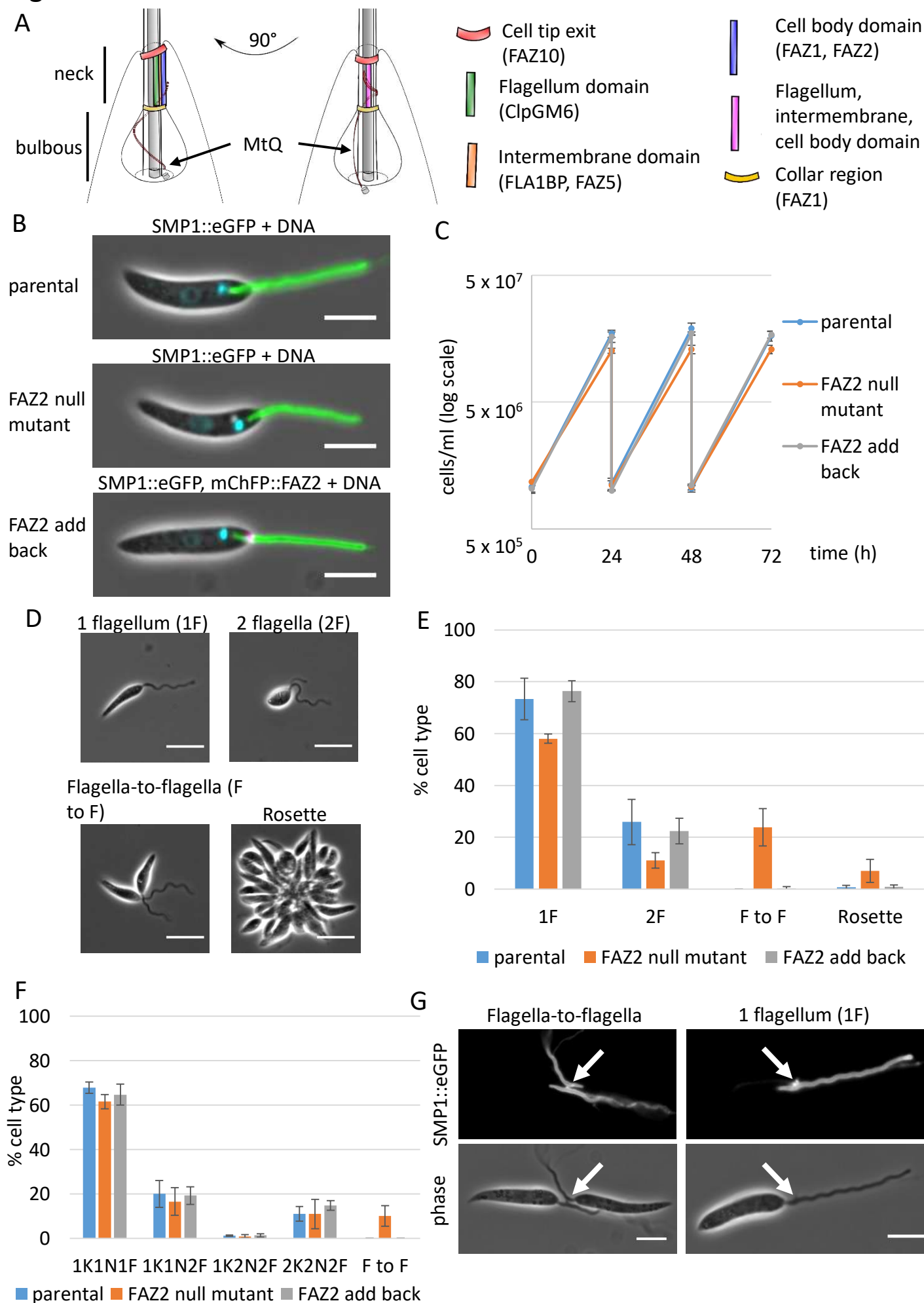


Figure 2

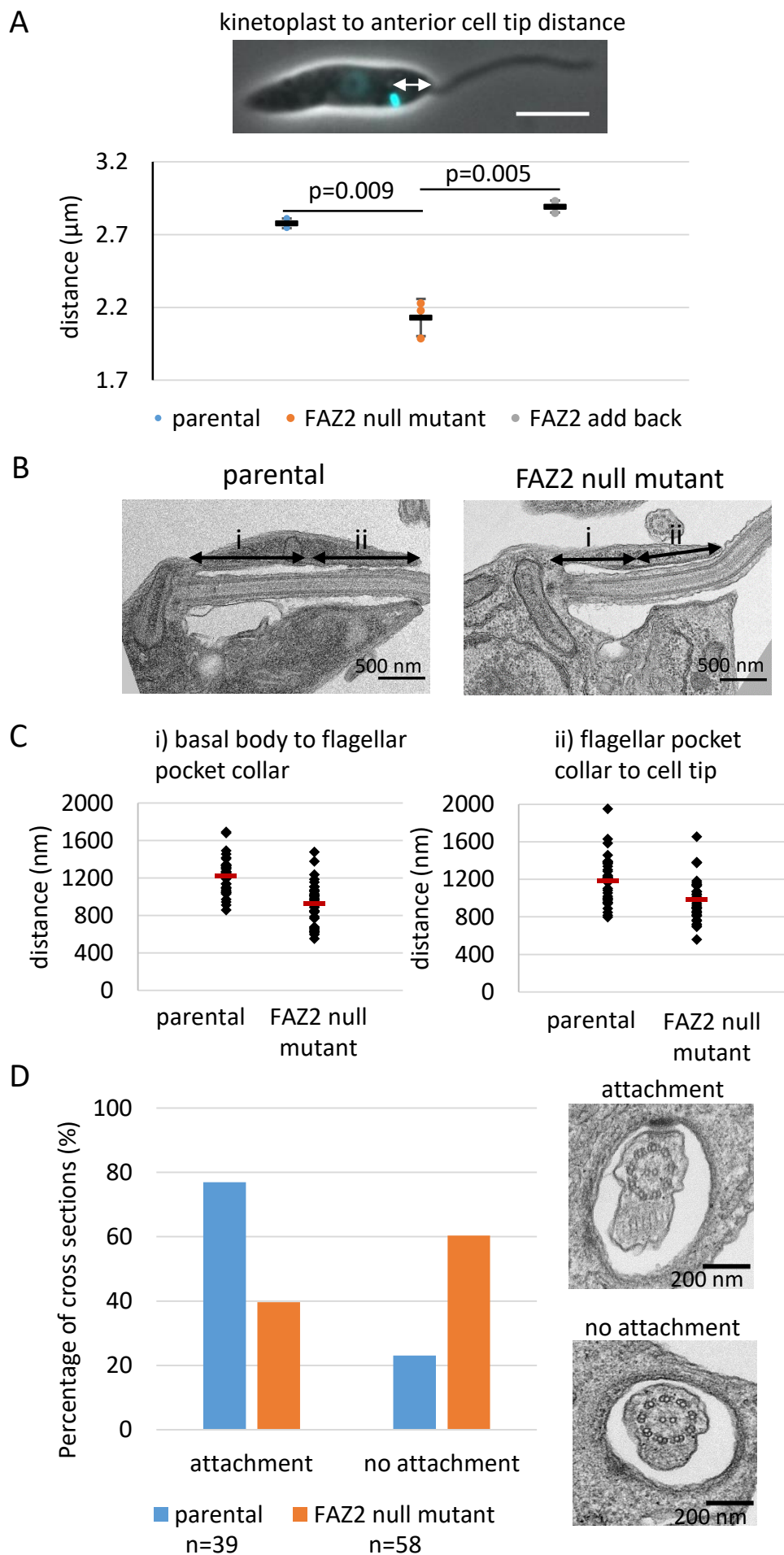
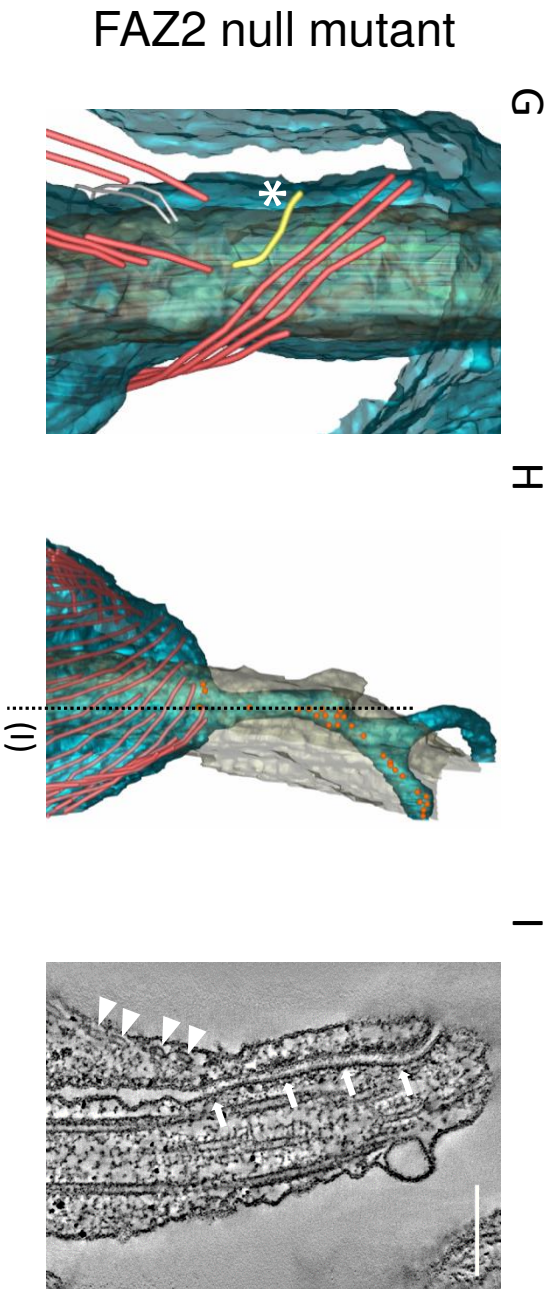
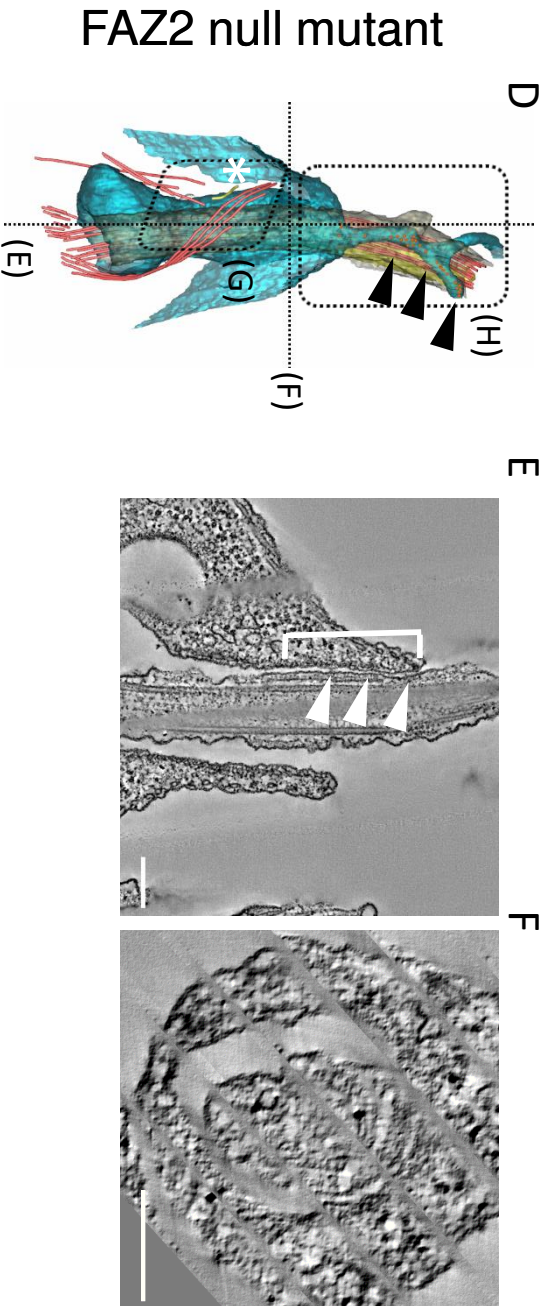
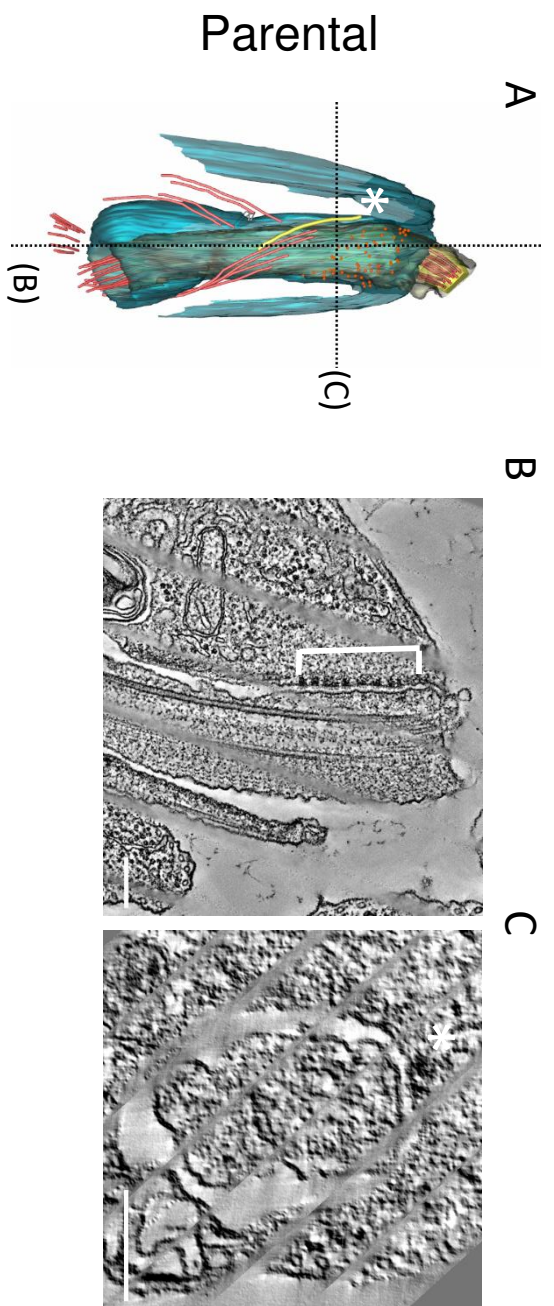


Figure 3



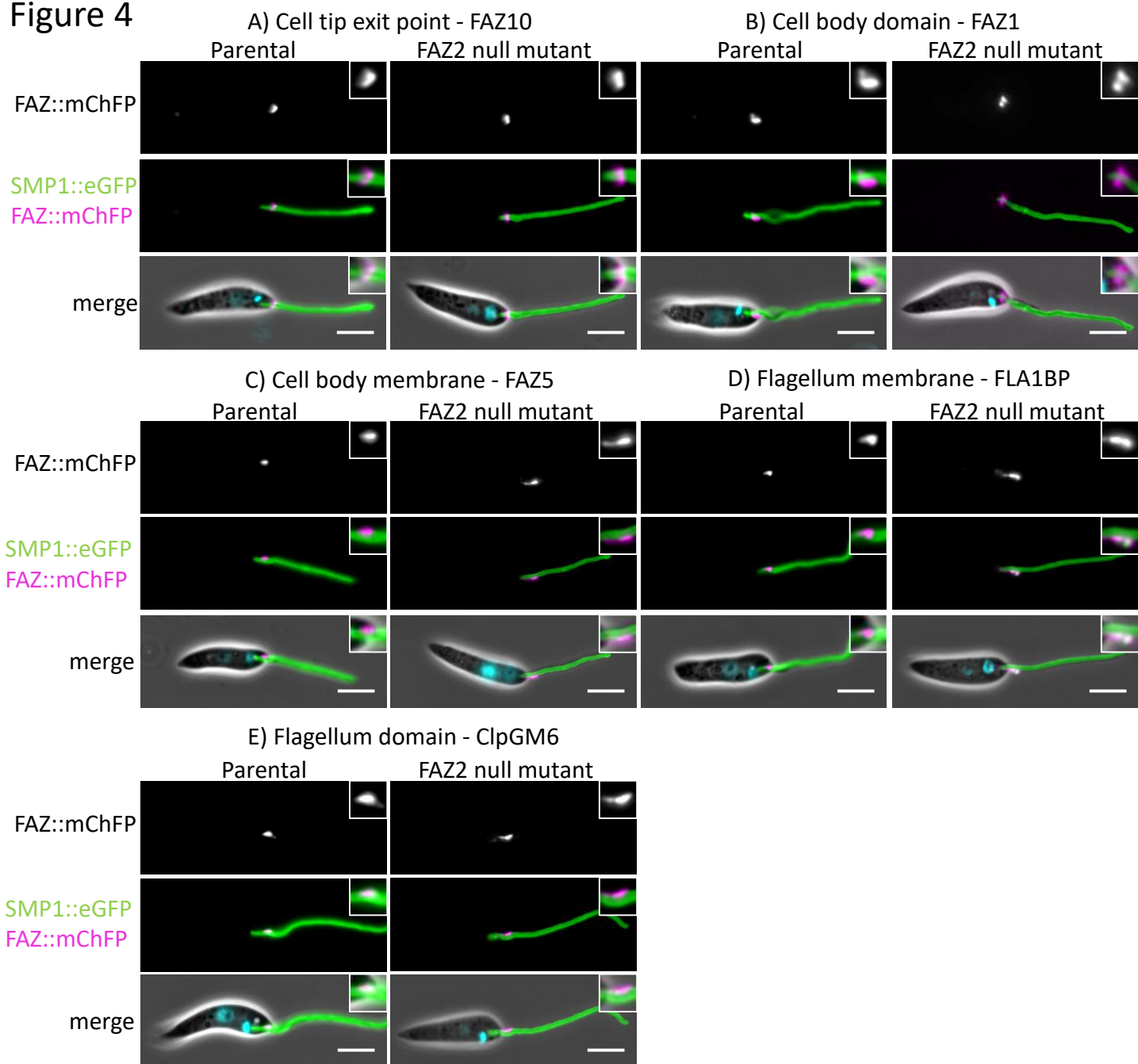
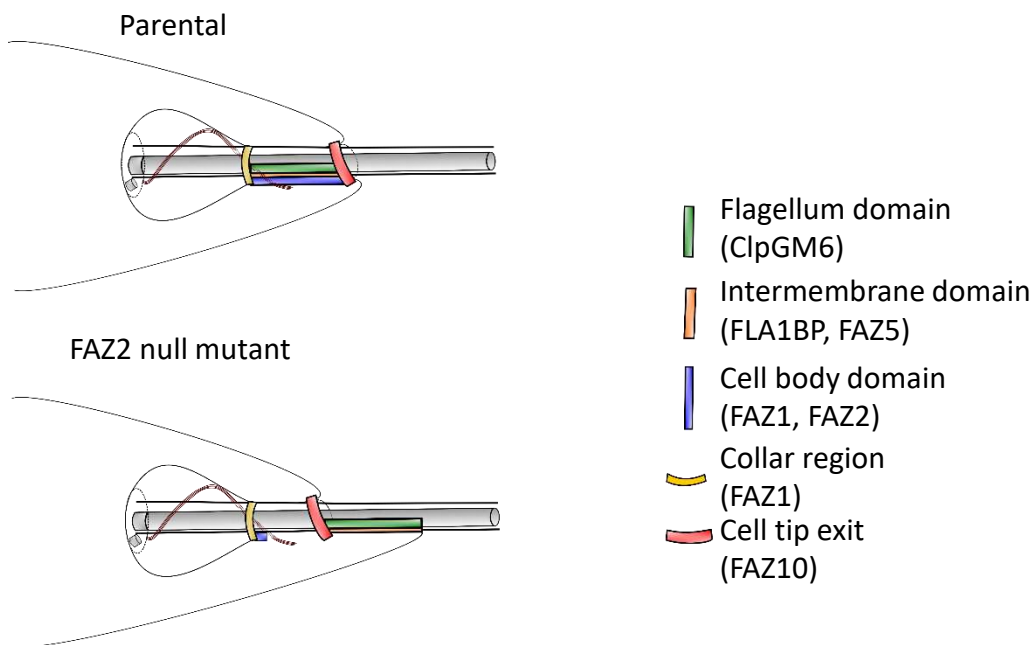
**Figure 4****F**

Figure 5

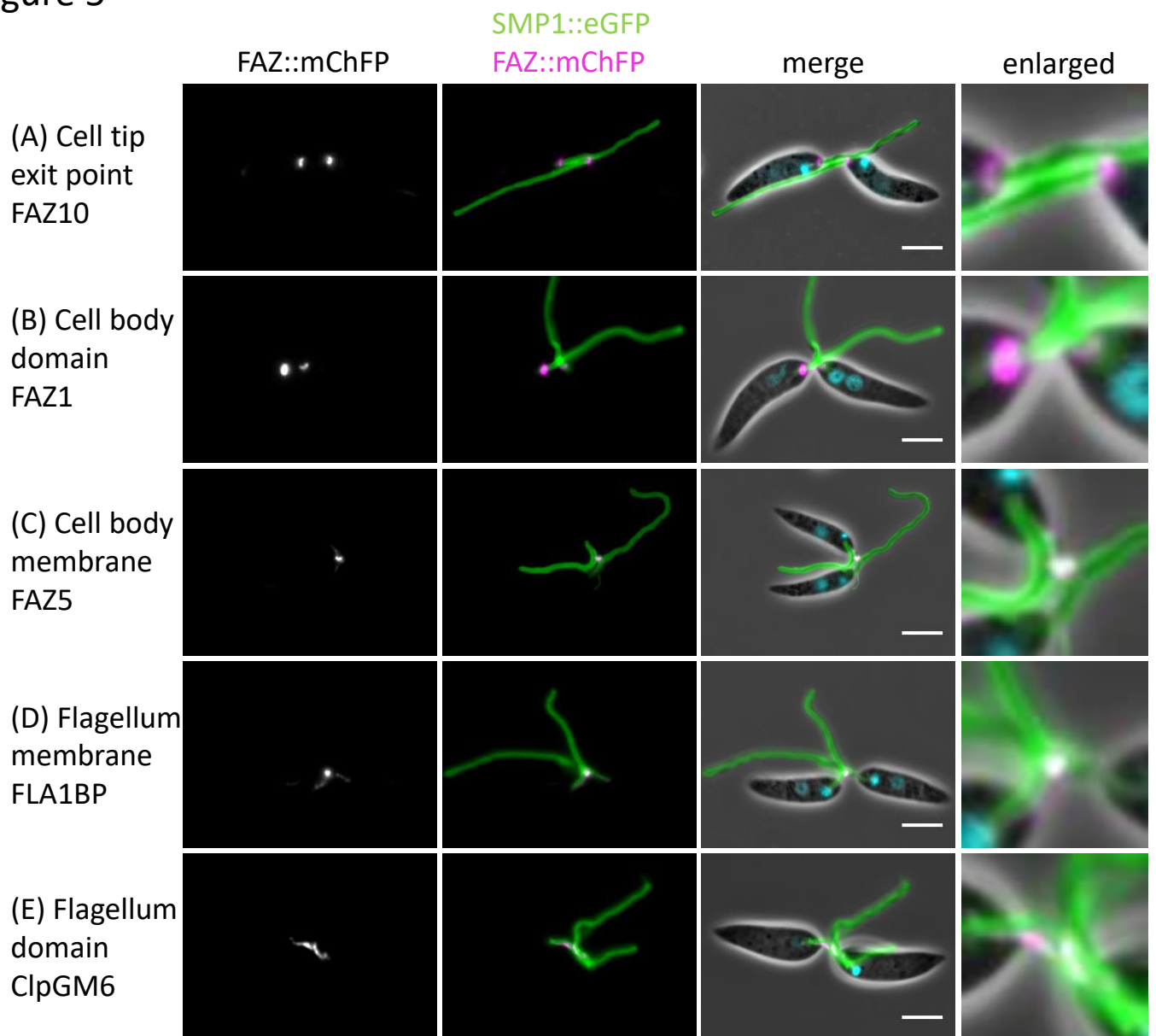
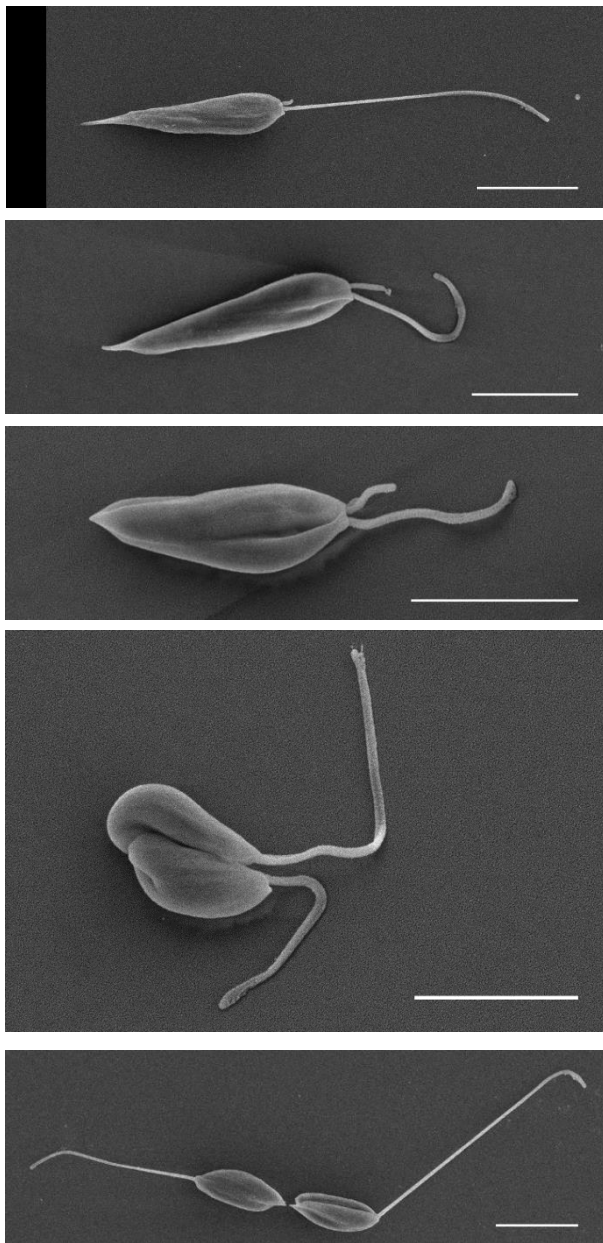




Figure 6

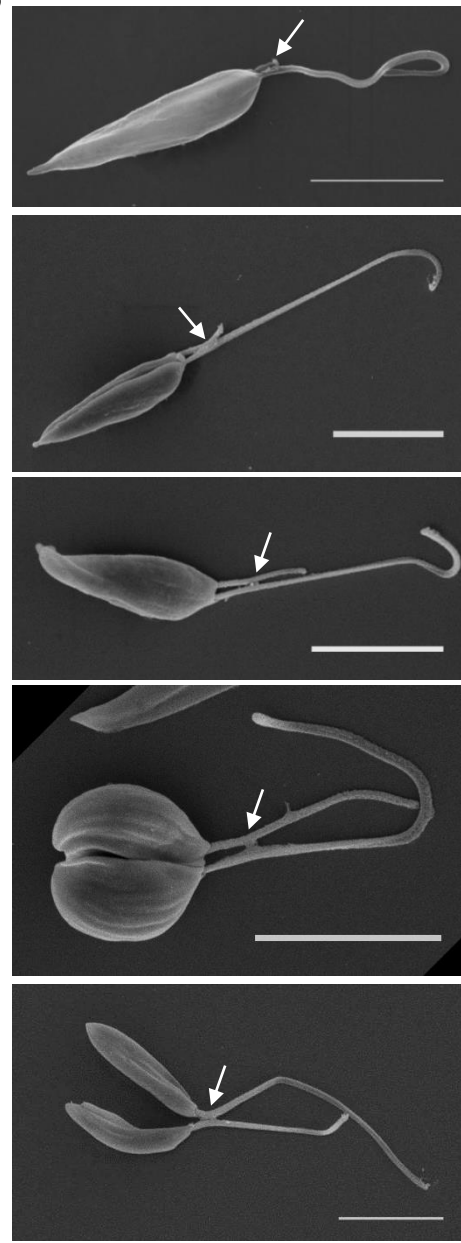
A

Parental

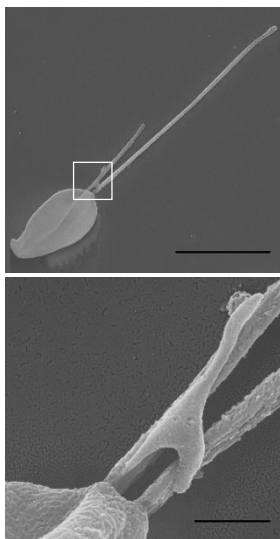


B

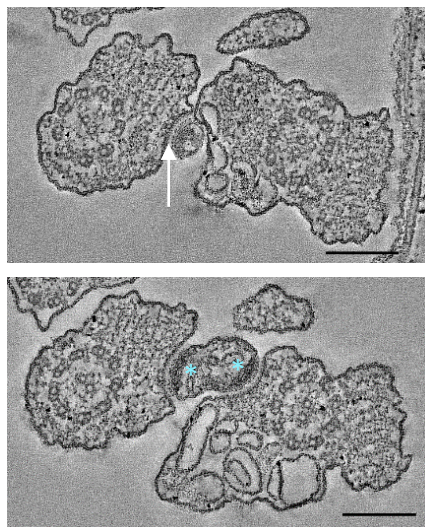
FAZ2 null mutant



C



D



E

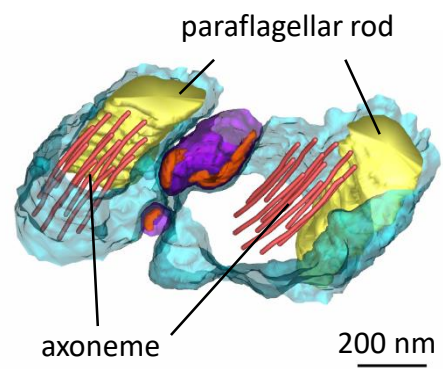


Figure 7

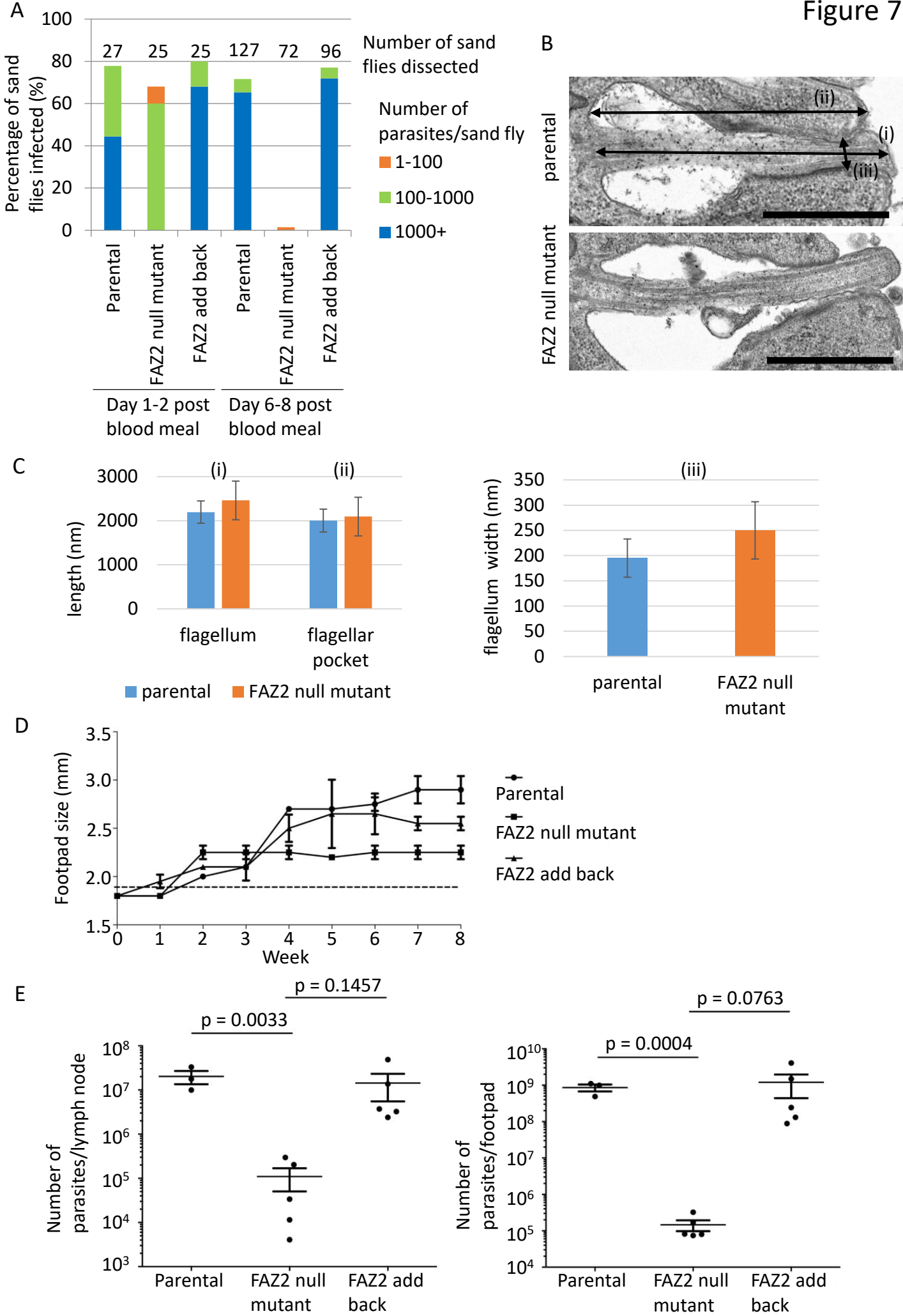
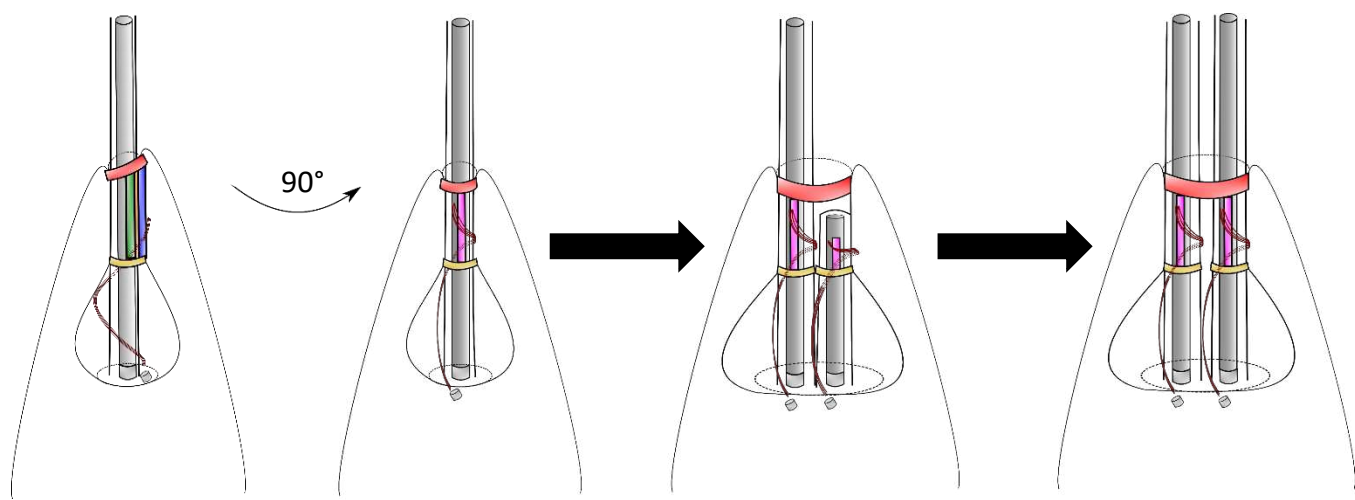
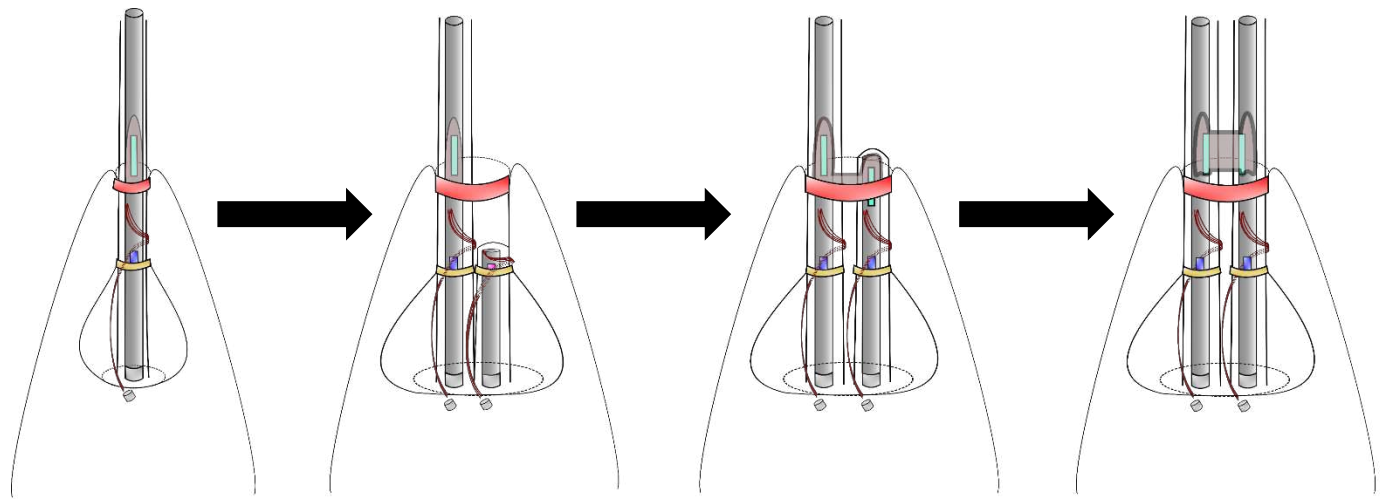


Figure 8

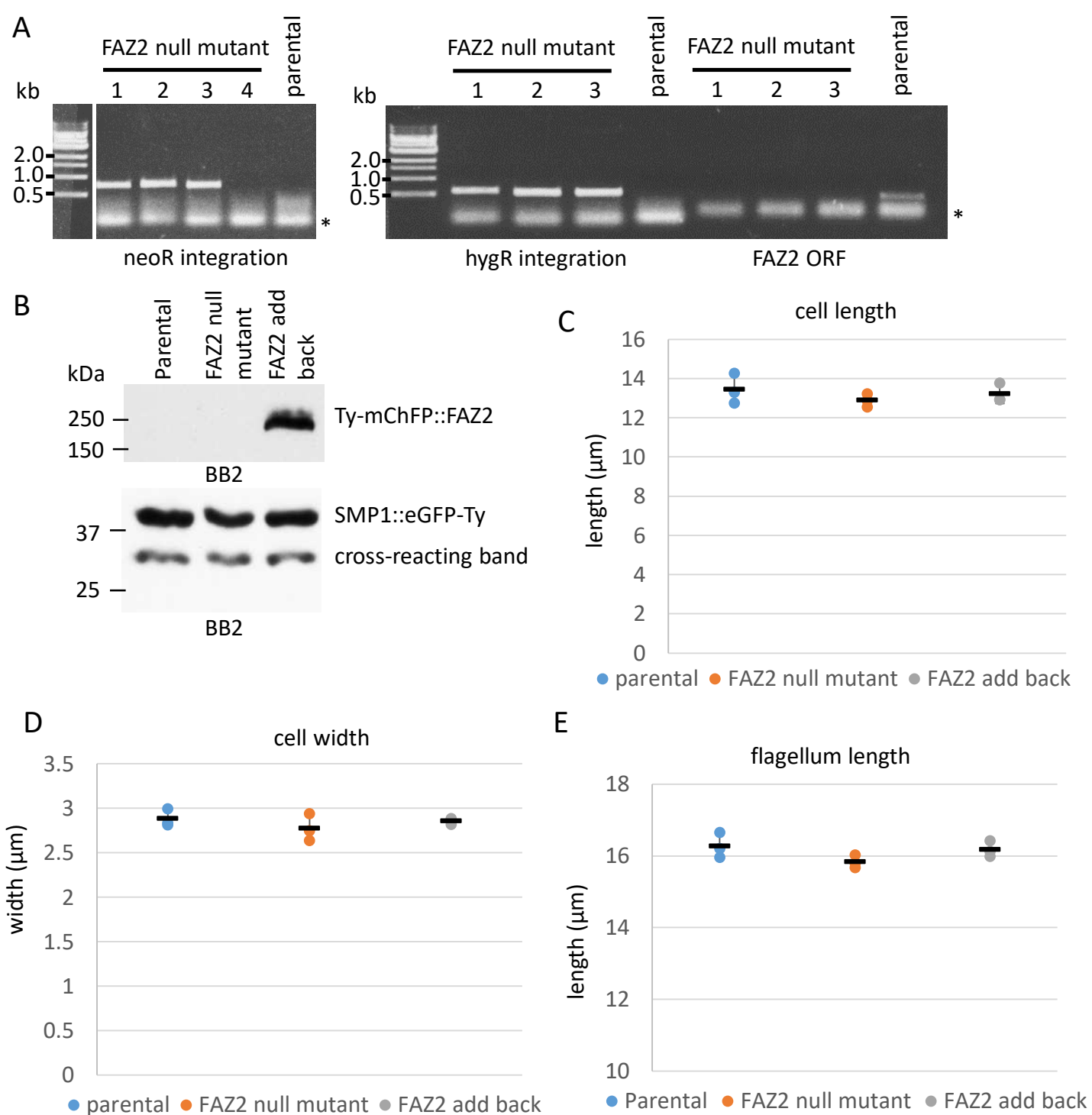
Parental



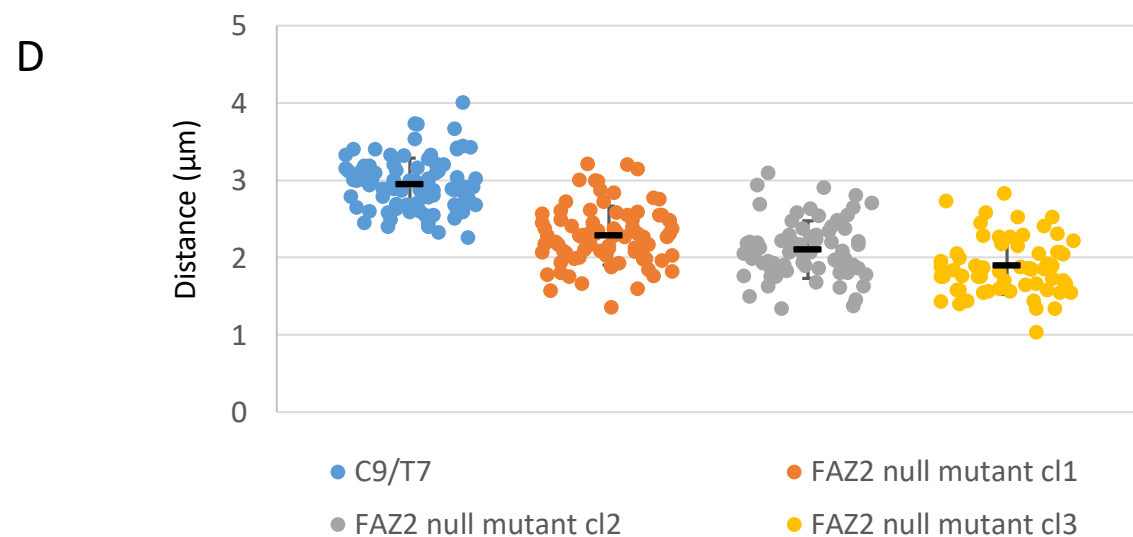
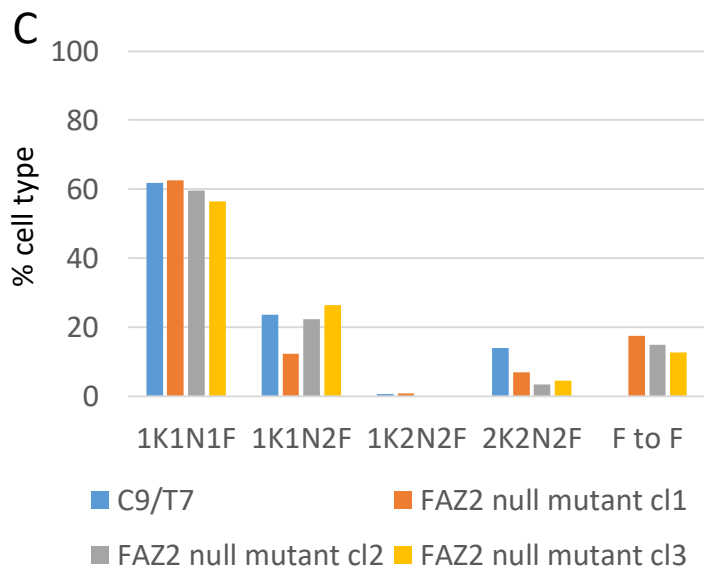
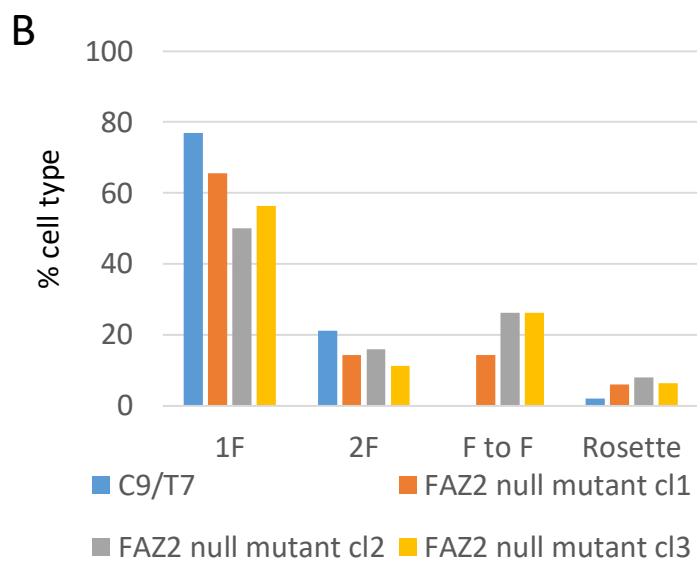
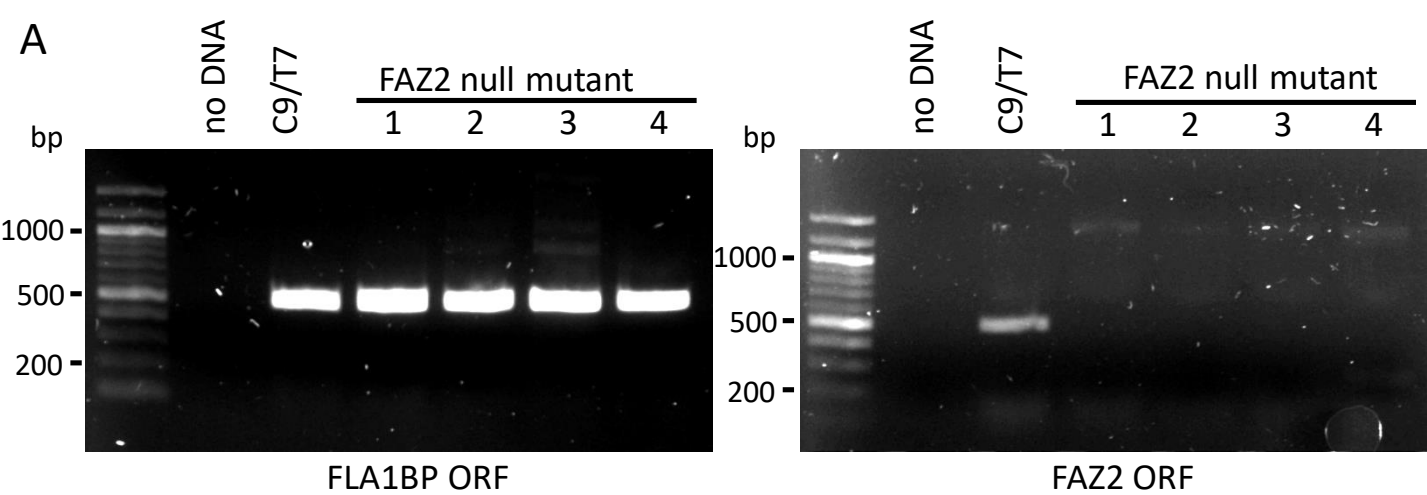
FAZ2 null mutant



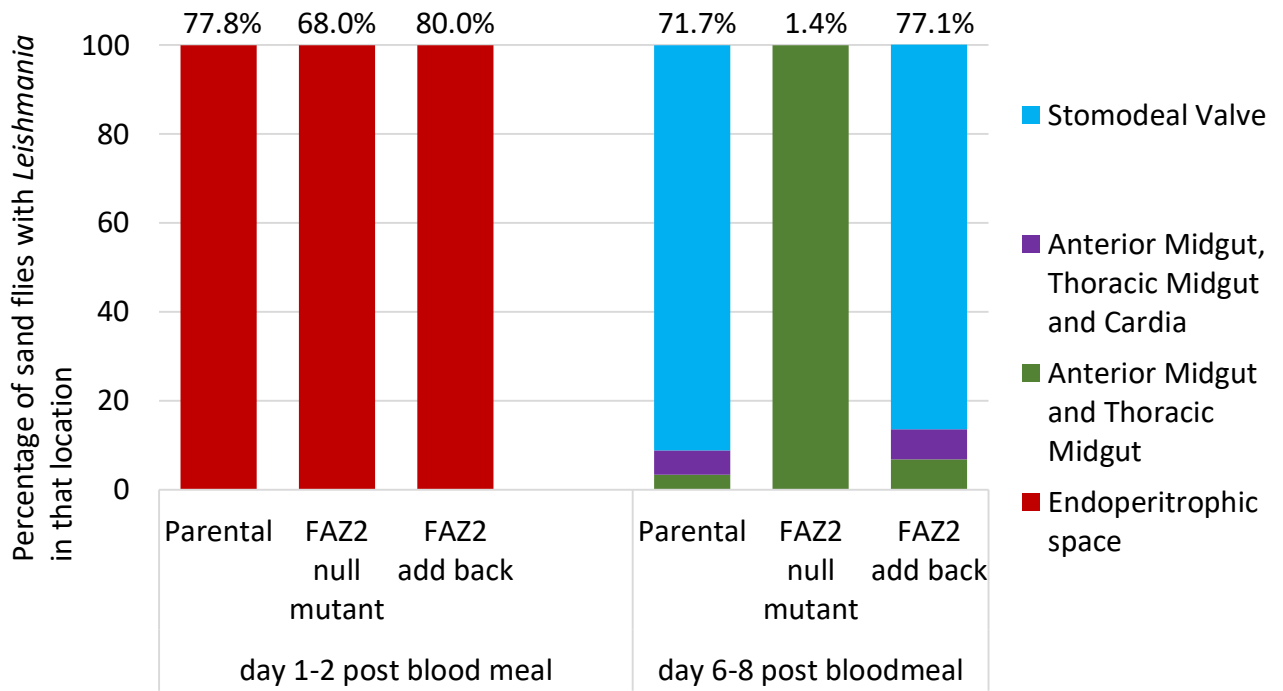
- Cell tip exit (FAZ10)
- Flagellum domain (ClpGM6)
- Intermembrane domain (FLA1BP, FAZ5)
- Cell body domain (FAZ1, FAZ2)
- Flagellum, intermembrane, cell body domain
- Collar region (FAZ1)
- Flagellum, intermembrane domain



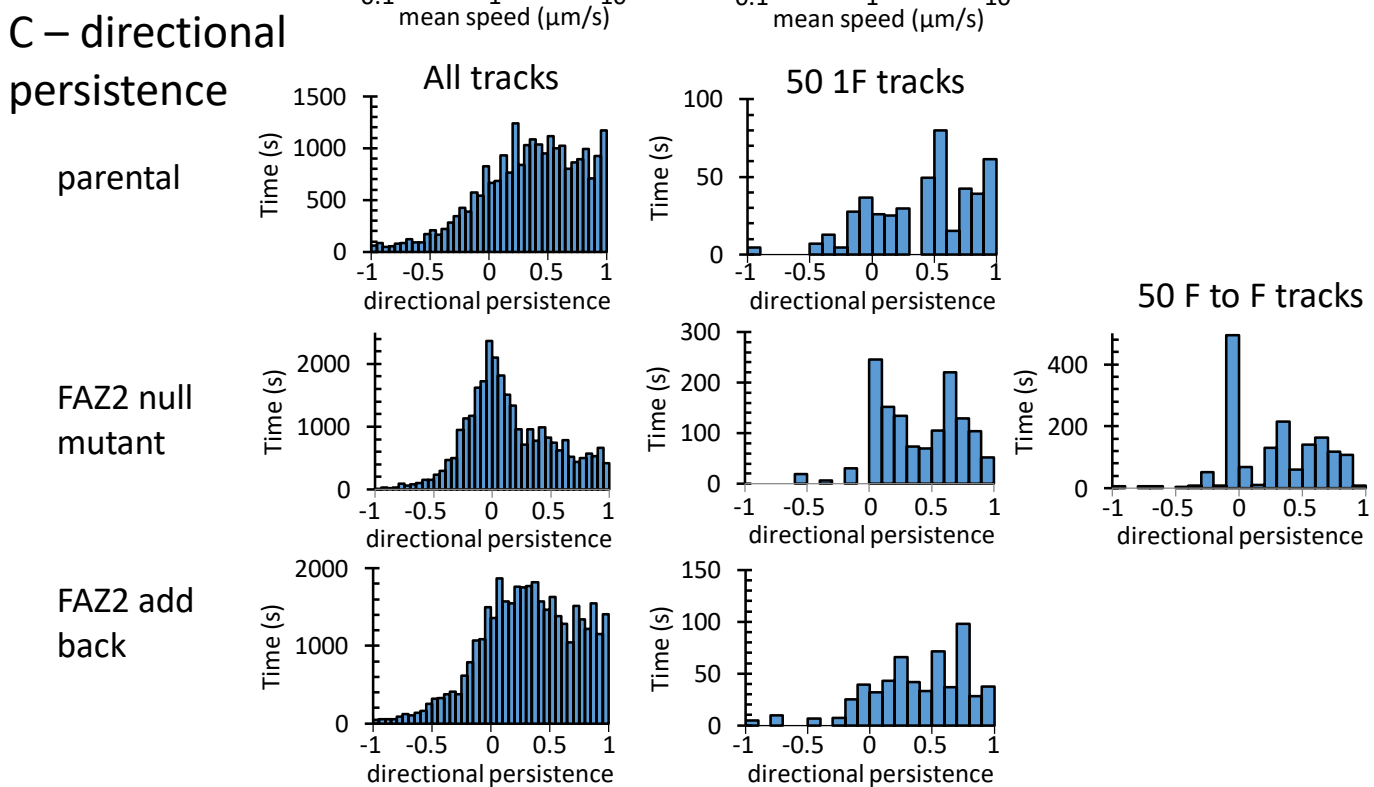
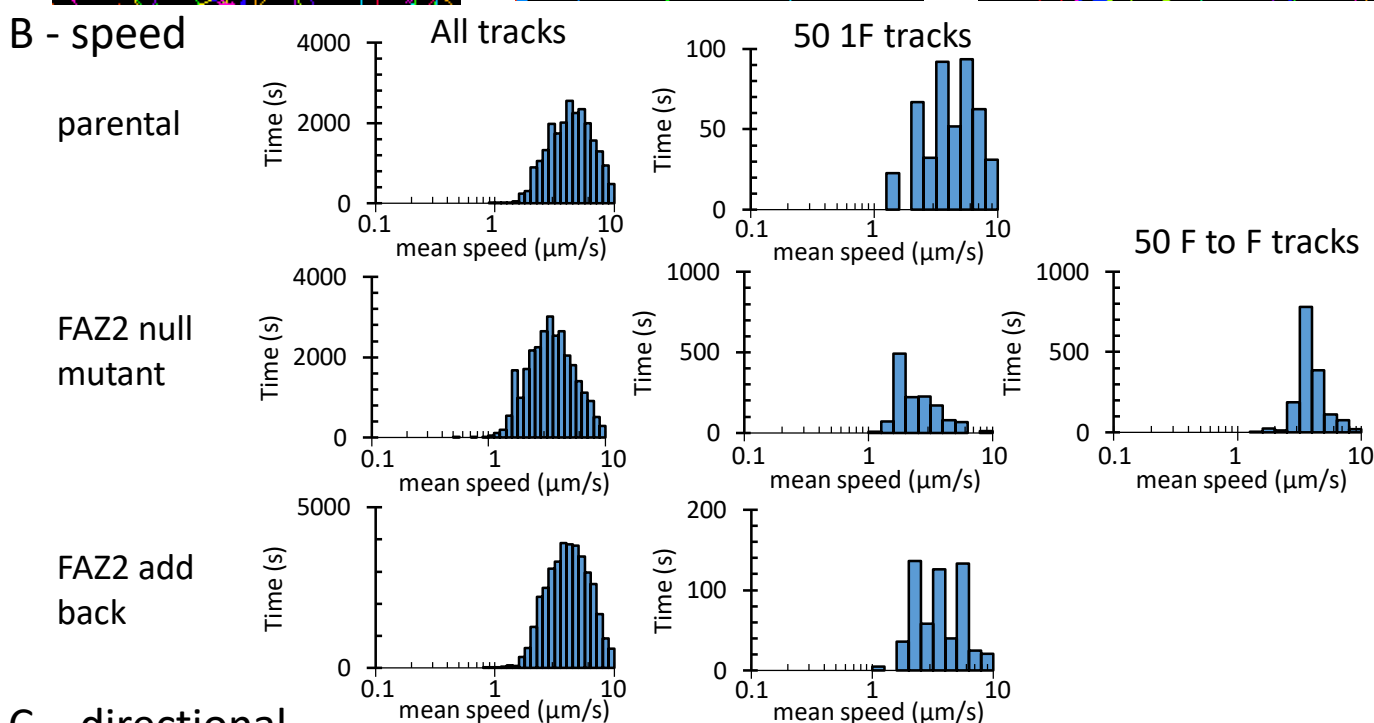
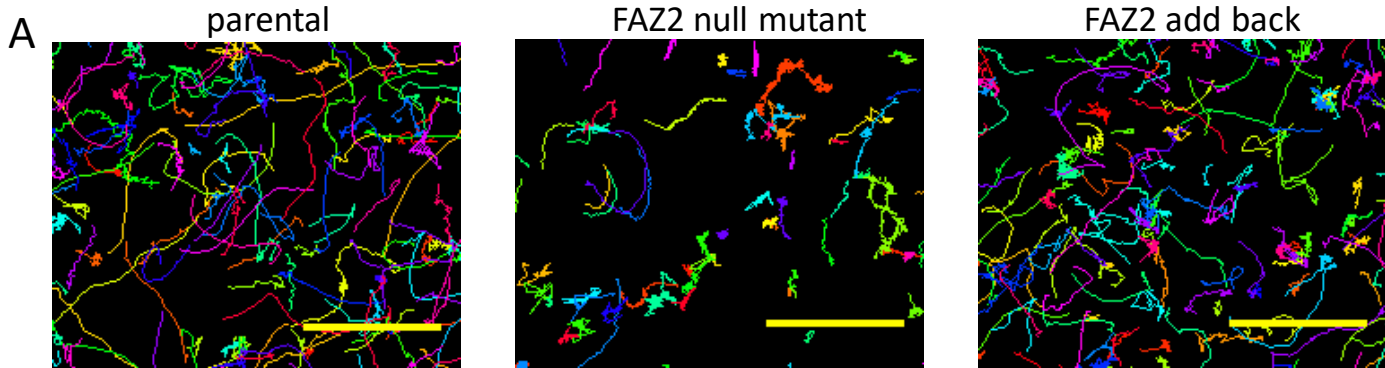
**S1 Fig** (A) Confirmation of FAZ2 gene deletion. gDNA from 4 null mutant clones and the parental cells was analysed by PCR. PCR confirmed that FAZ2 ORF was no longer present in the null mutant clones (1-3) and that the resistance markers had integrated correctly in clones (1-3). The neomycin resistance gene had not correctly integrated into clone 4 and this clone was discarded. FAZ2 null mutant clone 1 was used for all subsequent experiments. The lower less distinct band on the gel (\*) is likely be non-specific amplification of primer dimers. (B) Western blot confirming expression and expected size (174 kDa) of Ty-mChFP::FAZ2 using the BB2 antibody. The SMP1::eGFP-Ty and BB2 cross reacting band acted as a loading control. (C, D) Measurement of cell body length and width for parental, FAZ2 null mutant and FAZ2 add back cells. These measurements were done independently 3 times on at least 50 1K1N cells. The mean of each replicate is plotted as a circle with the mean and standard deviation of these individual means plotted as black lines. (E) Measurement of flagellum length for parental, FAZ2 null mutant and FAZ2 add back cells. These measurements were done independently 3 times on at least 100 1K1N cells. The mean of each replicate is plotted as a circle with the mean and standard deviation of these individual means plotted as black lines.



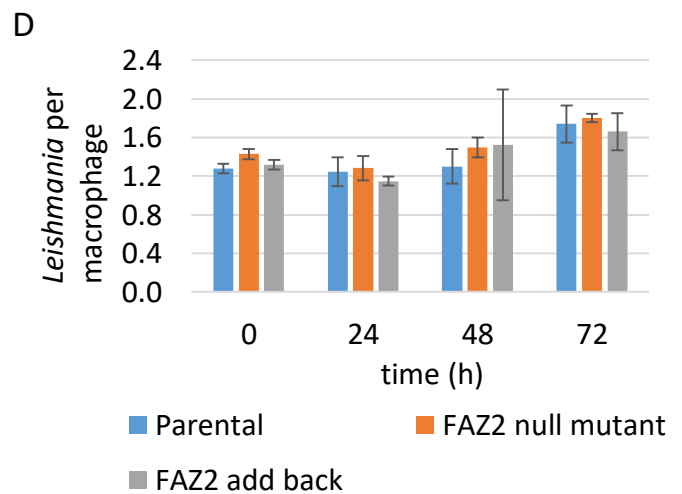
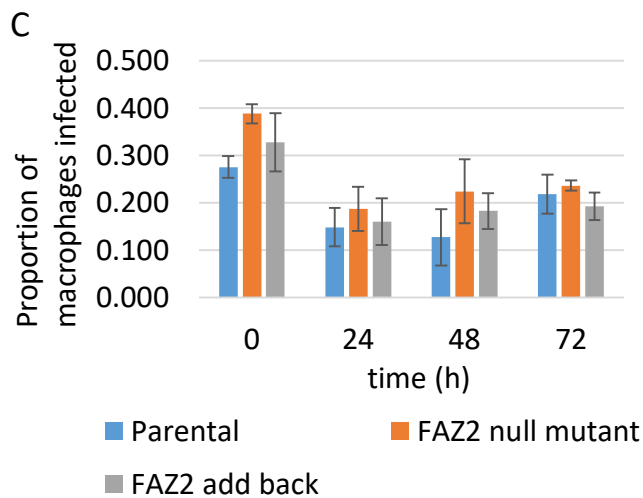
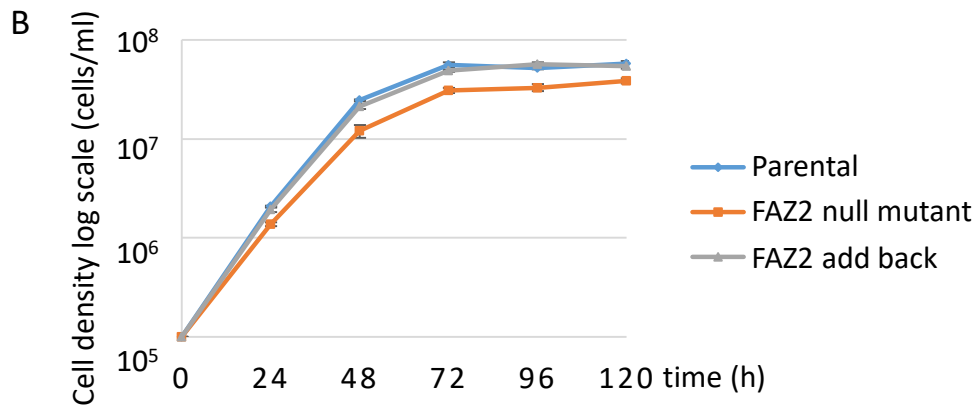
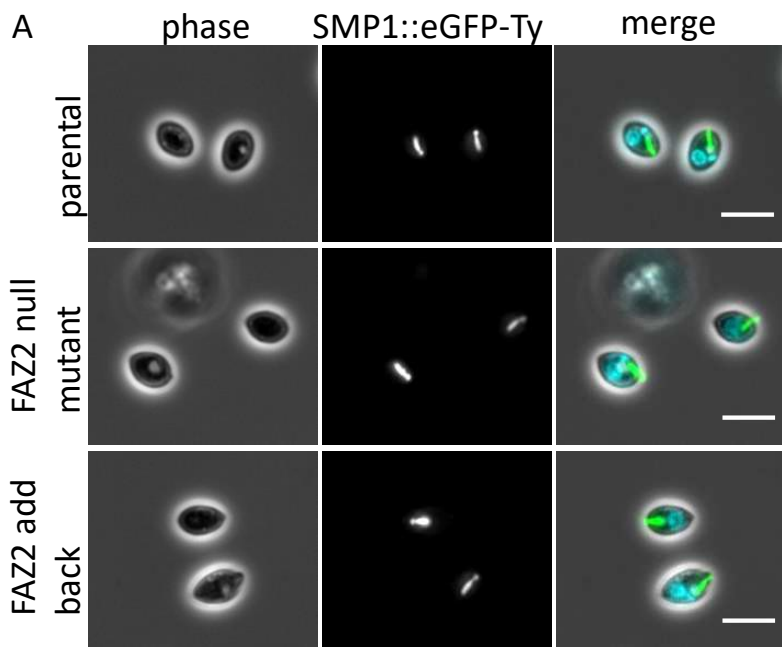
**S2 Fig** (A) Confirmation of FAZ2 gene deletion. gDNA from 4 null mutant clones and the parental cells was analysed by PCR. (B) Quantitation of cell types seen in culture for C9/T7 and FAZ2 null mutant clones. This experiment was performed once and for each cell line  $\geq 84$  cells were counted. (C) Cell cycle category counts for C9/T7 and FAZ2 null mutant clones. F – flagellum, K – kinetoplast, N – nucleus, F to F – two cells connected via their flagella. This experiment was performed once and for each cell line  $\geq 110$  cells were counted. (D) Measurement of the distance between the kinetoplast and the anterior end of the cell body for C9/T7 and FAZ2 null mutant clones. This experiment was performed once, each measurement is a coloured circle with the mean and s.d. plotted as black lines. For each cell line  $\geq 62$  cells were measured.



**S3 Fig** Migration of *Leishmania* in sand fly gut. Location of *Leishmania* parasites within infected sand flies at 1-2 and 6-8 days post blood meal. Stacked columns indicate the percentage of infected sand flies with parasites in various locations within the sand fly. FAZ2 null mutant was unable to migrate to the stomodeal valve. Percentage of infected flies for each cell line is indicated above each column. This is the combined data from two independent sand fly infection experiments.



**S4 Fig** (A) Swimming tracks from videomicroscopy of parental, FAZ2 null mutant and FAZ2 add back cells. Cells were imaged for 61 seconds with 512 images taken. Scale bar is 50  $\mu\text{m}$ . (B) Histograms of the mean speed for parental, FAZ2 null mutant and FAZ2 add back cells for all tracks imaged and for 50 1F cells and 50 F to F cells. (C) Histograms of the directional persistence for parental, FAZ2 null mutant and FAZ2 add back cells for all tracks imaged and for 50 1F cells and 50 F to F cells. The histograms and tracks are representative of two independent replicates.



**S5 Fig** (A) Images of axenic amastigotes of parental, FAZ2 null mutant and FAZ2 add back cells expressing SMP1::eGFP-Ty. Scale bar is 5  $\mu$ m. (B) *Leishmania* macrophage infections. Growth curve of parental, FAZ2 null mutant and FAZ2 add back cells to stationary phase - average of 3 replicates, mean  $\pm$  s.d is plotted. (C, D) Proportion of infected macrophages and the number of *Leishmania* per infected macrophage at 0, 24, 48, 72 hours post infection - 0 h time point is after 2 hours of infection and removal of cells not taken up. For each time point between 487-1074 macrophages were analysed. Mean  $\pm$  s.d. for 3 replicates is shown.