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1	A glycosylation mutant of <i>Trypanosoma brucei</i> links social
3	motility defects in vitro to impaired colonisation of tsetse in vivo
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25	Running title: Trypanosome social motility and fly transmission
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# 31 ABSTRACT

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> 34 Transmission of African trypanosomes by tsetse flies requires that the parasites 35 migrate out of the midgut lumen and colonise the ectoperitrophic space. Early 36 procyclic culture forms correspond to trypanosomes in the lumen; on agarose 37 plates they exhibit social motility, migrating en masse as radial projections from 38 an inoculation site. We show that an Rft1-/- mutant needs to reach a greater 39 threshold number before migration begins, and that it forms fewer projections 40 than its wild-type parent. The mutant is also up to 4 times less efficient at 41 establishing midgut infections. Ectopic expression of Rft1 rescues social motility 42 defects and restores the ability to colonise the fly. These results are consistent 43 with social motility reflecting movement to the ectoperitrophic space, implicate N-44 glycans in the signalling cascades for migration in vivo and in vitro, and provide 45 the first evidence that parasite-parasite interactions determine the success of 46 transmission by the insect host.

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#### 50 **INTRODUCTION**

51 Tsetse flies (Glossina spp) are the definitive hosts of the unicellular 52 parasite *Trypanosoma brucei*, while a variety of mammals can serve as 53 intermediate hosts. Different sub-species of T. brucei cause sleeping sickness in 54 humans and Nagana in domestic animals. The passage of *T. brucei* through the 55 tsetse fly was memorably described as a "journey fraught with hazards" (1) 56 because the majority of parasites are either eradicated or fail to complete the life 57 cycle. When trypanosomes are ingested by a tsetse fly as part of a blood meal 58 bloodstream forms differentiate into early procyclic forms in the midgut lumen. In 59 the first few days of tsetse infection there are two possible outcomes: the 60 parasites are either purged by the fly or they migrate through/around the 61 peritrophic matrix and colonise the ectoperitrophic space. Extraordinarily little is 62 known about this process: teneral (newly hatched) flies are more susceptible to 63 infection, most probably because the peritrophic membrane is not fully formed 64 and it is easier for parasites to gain access to the ectoperitrophic space (2). 65 There is evidence that several hundred parasites from the initial infectious blood 66 meal are founders of the population in the ectoperitrophic space (3). It is not 67 known, however, if these cross the peritrophic matrix individually or if they 68 migrate in groups. The majority of infections in tsetse do not proceed beyond the 69 midgut stage. Completion of the life cycle involves migration of a small number of 70 parasites to the salivary glands, expansion of the founder population as 71 epimastigote forms and the production of metacyclic forms that can be 72 transmitted to a new mammalian host (1, 3-5).

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73 The different life-cycle stages of *T. brucei* in the fly express characteristic 74 GPI-anchored glycoproteins that are present in several million copies per cell and 75 cover the entire surface. The early procyclic forms, which are detected in the fly 76 midgut for up to 7 days following fly infection (6), are characterised by the 77 presence of the GPI-anchored protein GPEET procyclin and lesser amounts of 78 EP procyclins (7). The late procyclic forms found in the ectoperitrophic space are 79 negative for GPEET, but continue to express EP1 and EP3 procyclin, both of 80 which are glycosylated (7). In addition to these major surface glycoproteins, 81 trypanosomes express other, less abundant membrane proteins, many of which 82 have the capacity to be modified by carbohydrates (8-11). 83 Early and late procyclic forms are usually cultured in liquid medium, but

84 they can also proliferate on a semi-solid surface (12). When early procyclic forms 85 are pipetted onto an agarose plate, the parasites first replicate at the inoculation 86 site and aggregate in groups. Upon reaching a threshold cell number, they 87 migrate outwards, resulting in the formation of radial projections or spokes (12, 88 13). This form of coordinated group movement has been termed social motility 89 (SoMo), based on similar behaviour in bacteria (13). Radial projections from two 90 communities growing on the same plate reorient to avoid encountering each 91 other, suggesting that the parasites produce and sense a repellent. Late 92 procyclic forms can also grow to high densities on plates. Although these do not 93 exhibit SoMo, they do produce substances that deflect the path of early procyclic 94 forms (12). It is evident that the coordination of mass movement on plates 95 requires cell-cell signalling, either through secreted factors or direct cell contact.

96 In this context it has recently been shown that the knockdown of either of two 97 adenylate cyclases at the flagellar tip results in a hypersocial phenotype, the 98 production of more radial projections (14, 15). Somewhat surprisingly, none of 99 the procyclins is required for SoMo (12). The three mutants so far found to be 90 defective are all motility mutants (13, 16).

101 Rft1 is an endoplasmic reticular protein involved in the conversion of 102 Man<sub>5</sub>GlcNAc<sub>2</sub>-PP-dolichol (M5-DLO) to M9-DLO, the precursor for N-linked 103 glycans (17-19). The protein is essential in yeast; in humans mutations have 104 been linked to congenital disease and glycosylation defects (20). Recently, an 105 *Rft1* knockout was generated in procyclic forms of *T. brucei* (19). The null mutant 106 accumulated M5-MLO, but had normal levels of mature dolichol-linked 107 oligosaccharide and was capable of glycosylating proteins. It also had a slightly 108 longer population doubling time than its parent (~15 h versus ~12 h in liquid 109 culture) and binding of the lectin concanavalin A (Con A) was reduced by 75%, 110 but no other defects were apparent. An addback expressing an ectopic copy of 111 *Rft1* showed wild-type levels of Con A-binding, confirming that the phenotype 112 was linked to the presence or absence of the gene (19). N-linked glycosylation is 113 known to play a pivotal role in in the folding, quality control, stability and function 114 of surface and secreted proteins (21, 22). It can also be a determinant of signal 115 transduction and host-pathogen interactions (23) and has been implicated in 116 density sensing and adhesion during the development and swarming behaviour 117 of Dictyostelium (24-26).

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118 It has been a matter of some debate whether SoMo is a phenomenon that 119 only occurs in culture or if it is a manifestation of an event that occurs in vivo 120 (27). Based on its restriction to early procyclic forms, it has been hypothesised 121 that SoMo reflects the migration from the midgut lumen to the ectoperitrophic 122 space (12). We show that the Rft1 null mutant is compromised both in its ability 123 to perform SoMo and in the establishment of midgut infections. This provides the 124 first evidence that SoMo reflects a specific event in vivo and highlights the 125 importance of parasite-parasite interactions in allowing them to colonise their 126 host.

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#### 128 MATERIALS AND METHODS

**Trypanosomes.** The parental strain *T. b. brucei* Lister 427, *Rft1 -/-* null mutant and addback were described previously (19). Early procyclic forms were cultured in SDM-79 containing 10% foetal bovine serum and 20 mM glycerol or on plates containing the same medium supplemented with 0.4% agarose (12).

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134 Flow cytometry. This was performed as described (28). Briefly, cells were fixed

135 with 4% paraformaldehyde and 0.2% glutaraldehyde for 20 minutes at room

136 temperature and were then blocked for 1 h with 4% bovine serum albumin.

137 Immunostaining with anti-EP and GPEET antibodies was performed as described

138 by Vassella et al. (6). The secondary antibodies Alexa-Fluor 488 goat anti-rabbit

139 and Cy3 goat anti-mouse (Invitrogen) were used at dilutions of 1:1000. Ten

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140 thousand cells per sample were analysed using a FACscalibur (BD Biosciences)

141 and analysed with FlowJo.

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143 Plating and social motility assay. Plates were poured as described and used within 24 h (12). Following inoculation, the plates were sealed with parafilm and 144 145 incubated at 27° with 5% CO<sub>2</sub>. To determine the cell number at the point when 146 migration started, cells were spotted onto the surface of an agarose plate and 147 incubated as described above. The inocula for the wild type and addback were 2x10<sup>5</sup> cells and the inoculum for the Rft1 null mutant was 4x10<sup>5</sup>. Plates were 148 inspected every 8 - 12 hours. At the point when radial projections became visible 149 150 trypanosomes were washed from the plate in 1 ml of serum-supplemented SDM-151 79 and counted using a haemocytometer. Only viable cells were scored. With 152 these inocula the majority of cells remained viable. Plates were photographed as 153 described (12).

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Fly infections. *Glossina morsitans morstinans pupae* were obtained from the Department of Entomology, Slovak Academy of Science, Bratislava. Infection of teneral flies and grading of infections were performed as described (29). The parental line was tested 3 times, and the knockout and addback twice, in independent experiments.

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161 **Imaging of trypanosomes in liquid culture**. Logarithmically growing cultures 162 were diluted to  $4 \times 10^6$  cells ml<sup>-1</sup> in complete medium and 10µl were transferred

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to a Neubauer haemocytometer counting chamber. Images were taken every
0.5s for 20 seconds with a Leica DFC360FX monochrome CCD (charge-coupleddevice) camera mounted on a Leica DM5500 B microscope with a 20x objective
using LAS AF software (Leica). Movies were generated with ImageJ.

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## 169 **RESULTS AND DISCUSSION**

170 We first compared the ability of the Rft1 null mutant (19) and its wild-type 171 parent to perform SoMo on agarose plates. Before embarking on these 172 experiments we determined the number of early (GPEET-positive) procyclic 173 forms in each population since only early procyclic forms show SoMo (12). The 174 parent, knockout and addback were 94.4, 87.6 and 91.9% GPEET-positive, 175 respectively, and expressed similar levels of GPEET and EP procyclins (Figure 176 1A), indicating that underglycosylation did not cause obvious changes in surface 177 architecture. Initially we had difficulty in culturing the null mutant on agarose 178 plates as the cells tended to die. If a larger inoculum was used the mutant 179 survived and replicated, but in contrast to the parental control, colonies did not 180 produce spokes over a 5-day period (Figure 1B). If plates with the null mutant 181 were incubated for longer, radial projections eventually formed, but the numbers 182 were consistently lower than for the parental line (Figure 2A and B). The addback 183 derived from the knockout (19) did not exhibit difficulties in growing on plates, 184 and formed similar numbers of projections as the wild type and at a similar rate 185 (Figure 2A and B). This confirmed that the phenotypic differences were due to

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187	that the cells needed to reach a threshold density on plates, a trivial explanation
188	would be that the knockout took longer to generate this number of cells. We
189	therefore performed a series of experiments in which the number of viable cells
190	was determined at the point when colonies began to form projections (Figure 2C
191	and D). The threshold numbers for wild-type <i>T. brucei</i> (1.85 $\pm$ 0.47 x10 <sup>6</sup> ) and the
192	addback (1.64 $\pm 0.17 \text{ x10}^6$ ) were in excellent agreement with the number
193	previously determined for strain AnTat 1.1 (12). In contrast, the threshold for the
194	knockout was 3.86 $\pm 0.83 \times 10^6$ . Taken together with the lower number of radial
195	spokes, this implies that cells lacking Rft1 either produce lower amounts of the
196	factor stimulating migration (or a less active form of it), or are not as receptive to
197	the signal. As mentioned above, trypanosomes that show motility defects in liquid
198	culture are compromised in their ability to migrate on plates (13, 16). The motility
199	of the Rft1 null mutant was normal, however (Supplemental movies 1-3). In
200	addition, it could still produce and sense the repellent(s) produced by other
201	communities, resulting in projections reorienting and avoiding each other (Figure
202	2B). In this respect there was no indication that it differed from the wild type.
203	We next investigated whether a lack of Rft1 affected the ability of the
204	knockout to establish midgut infections. To date, a knockout of GPI8, the
205	transamidase that transfers the preformed GPI anchor to protein precursors, is
206	the only mutant to show pronounced defects in infecting the midgut (30, 31).
207	Teneral flies were infected with the parental line or one of the mutants and
208	monitored for the prevalence and intensity of midgut infections. Infections were

Rft1. Bearing in mind that the mutant replicated more slowly than its parent, and

209 graded into 4 categories (negative, weak, intermediate and heavy) as described 210 previously (29). A first experiment was performed with the wild type and the null 211 mutant; a second experiment also included the addback mutant. Flies were 212 dissected 3 and 14 days post infection (dpi; Figure 4). Dissections at 3 dpi 213 determined whether trypanosomes could survive in the fly at all, while those at 14 214 dpi determined whether they had succeeded in establishing an infection. In both 215 experiments the wild-type parental line and the knockout gave very similar 216 profiles at 3 dpi, indicating that *Rft1* was not crucial for survival of early procyclic 217 forms in the midgut lumen. At 14 dpi the knockout showed a 2-4 fold lower 218 prevalence of established infections than the wild type. When infections did 219 occur, however, their intensities were similar (mostly heavy infections). This 220 indicates that if the null mutants manage to reach the ectoperitrophic space they 221 can proliferate normally. Once again, expression of the ectopic copy in the 222 addback rescued the phenotype, confirming that Rft1 influences the ability of 223 trypanosomes to colonise the midgut and/or resist clearance by the fly. 224 In conclusion, we provide the first evidence for a link between the ability of 225 parasites to perform SoMo and to establish midgut infections, consistent with 226 SoMo reflecting the migration from the lumen to the ectoperitrophic space. 227 Furthermore, these results implicate N-linked glycans in the biogenesis, stability 228 or activity of the migration factor(s) and/or components of the signalling cascade 229 for SoMo, and the function of factors promoting colonisation in vivo. The N-230 glycans on the EP procyclins and the transmembrane protein PSSA-2 can be 231 excluded, as these proteins are neither essential for SoMo (12) nor for

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232 establishing midgut infections (6, 9). However, both adenylate cyclases that 233 regulate SoMo are glycosylated (14). Moreover, there are a number of surface-234 associated enzymes (10, 11), nutrient transporters (8) and flagellar components 235 (32, 33) that are (potentially) N-glycosylated and might conceivably play a role in 236 SoMo, as well as additional adenylate cyclases that are differentially expressed 237 between early and late procyclic forms (12). 238 239 ACKNOWLEDGMENTS 240 This work was funded by grants from the Howard Hughes Medical Institute (no.

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#### 371 FIGURE LEGENDS

## 373 Figure 1

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- 374 Procyclin expression and social motility phenotypes.
- 375 A. Surface expression of EP and GPEET procyclin on wild-type 427, Rft1
- 376 knockout (KO) and addback cells. Trypanosomes were co-stained with
- 377 antibodies against EP and GPEET and analysed by flow cytometry. The upper
- 378 left panel is a negative control in which the primary antibodies were omitted.
- 379 B. Rft1 knockout (left) and wild-type trypanosomes (right) were inoculated onto
- the surface of a 0.4% agarose plate. The plate was photographed after 5 days
- incubation at 27°.
- 382 WT: wild type; KO: Rft1 knockout.
- 383

## 384 Figure 2

- 385 The *Rft1* knockout (KO) forms fewer projections and requires a higher threshold
- 386 density than wild type (WT) and addback trypanosomes.
- 387 A. Numbers of projections formed by individual communities on agarose plates.
- 388 B. Representative examples of plates, showing the number of projections and
- 389 that the knockout is capable of producing and responding to repellents.
- 390 C. Cell number at the point that projections start to form.
- 391 D. Representative plates for the data depicted in C.
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# **Figure 3**

- 397 The *Rft1* knockout produces a lower number of midgut infections. Flies were
- 398 dissected 3 and 14 days post infection (dpi) and graded for intensity of infections.
- 399 A. Experiment 1. B. Experiment 2. In both cases the difference between the wild
- 400 type and knockout at 14 dpi were statistically significant (one tailed Fisher's exact
- 401 test).
- 402 WT: wild type; KO: *Rft1* knockout.
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KO WT

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Figure 2 Imhof et al.

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Figure 3 Imhof et al.