Forward motility is essential for trypanosome infection in the tsetse fly

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Summary
African trypanosomes are flagellated protozoan parasites transmitted by the bite of tsetse flies and responsible for sleeping sickness in humans. Their complex development in the tsetse digestive tract requires several differentiation and migration steps that are thought to rely on trypanosome motility. We used a functional approach in vivo to demonstrate that motility impairment prevents trypanosomes from developing in their vector. Deletion of the outer dynein arm component DNAI1 results in strong motility defects but cells remain viable in culture. However, although these mutant trypanosomes could infect the tsetse fly midgut, they were neither able to reach the foregut nor able to differentiate into the next stage, thus failing to complete their parasite cycle. This is the first in vivo demonstration that trypanosome motility is essential for the accomplishment of the parasite cycle.

Introduction
Trypanosoma brucei are protozoan parasites responsible for human African trypanosomiasis or sleeping sickness, a neglected tropical disease in Central Africa (Simarro et al., 2008; Chappuis et al., 2010). They also cause nagana in cattle, a similar disease with major socio-economic consequences (Brun et al., 2009). Trypanosomes proliferate as extracellular parasites in the bloodstream of their mammalian host and can cross the blood-brain barrier to provoke severe neurological symptoms causing death in the absence of treatment (Brun et al., 2009). Trypanosomes are transmitted by the bite of the tsetse flies, which become vectors when they consume a blood meal from an infected mammal. To complete their life cycle, trypanosomes initially present in the posterior midgut of the fly have to reach the tsetse salivary glands in order to transform into infective parasites (Vickerman, 1985; Peacock et al., 2007; Oberle et al., 2010; Rotureau et al., 2012). This is not direct and requires several proliferation, differentiation and migration steps that take place for 2–3 weeks in a strictly defined chronological order in five distinct fly tissues (Vickerman, 1985; Van Den Abbeele et al., 1999; Sharma et al., 2009; Oberle et al., 2010).

Trypanosomes found in the mammalian blood circulation are trypomastigote forms, where the basal body of the flagellum, that is linked to the DNA of the single mitochondrion called the kinetoplast (Robinson and Gull, 1991), is present at the far posterior end of the cell. Several hours after their ingestion by the fly, bloodstream trypomastigotes differentiate into procyclic trypomastigotes and proliferate in the posterior midgut of the fly. From there, part of the procyclic trypomastigote population cross the peritrophic matrix, elongate into mesocyclic trypomastigotes and migrate anteriorly along the tsetse alimentary tract, through the anterior midgut and the proventriculus to reach the foregut (Vickerman, 1985). This first crucial migration step is accompanied by a differentiation of the mesocyclic trypomastigotes into dividing epimastigotes, where the basal body of the flagellum is found in an anterior position relative to the nucleus (Van Den Abbeele et al., 1999; Sharma et al., 2008; Rotureau et al., 2011). All these parasite stages possess a flagellum that could be essential for the completion of the parasite cycle (Rotureau et al., 2011). Since the primary function of the T. brucei flagellum is for motility, it is reasonable to expect that trypanosome motility is required for parasite development.

Different modes of locomotion have been described in unicellular organisms such as gliding for Toxoplasma tachyzoites in the human gut (Heintzelman, 2006), crawling for the green algae Chlamydomonas (Heintzelman, 2006), amoeboïd movements for the ubiquitous parasite of the human intestine Entamoeba (Tavares et al., 2000), passive diffusion for Plasmodium sporozoites in the Anopheles haemolymph (Hillyer et al., 2007), or piggy-backing for Leishmania infantum amastigotes transported to the viscera via the mononuclear phagocyte system (Kaye and Scott, 2011). Trypanosomes are efficient
swimmers in liquid culture medium: their mobility results from the beating of their flagellum that is generated by dynein motors distributed along the axoneme (review in Hill, 2010). RNAi silencing of the paragl卖ellar rod protein PFR2 in monomorphic bloodstream trypanosomes intraperitoneally injected in mice caused their rapid clearance (Griffiths et al., 2007). According to the authors, this would be explained by the reduction of parasite motility that was monitored in parallel in vitro. However, to our knowledge, there is no other evidence of the importance of swimming in trypanosome in vivo, especially in the tsetse fly vector.

We reasoned that the dynein intermediate chain (DNAI1) of the flagellum axoneme could be a good target to test whether forward swimming is essential for the accomplishment of the parasite cycle. Inducible RNAi expression in cultured trypanosomes led to the loss of outer dynein arms along the axoneme microtubule doublets, resulting in the blockage of the tip-to-base wave in the flagellum (Branche et al., 2006). These parasites were still able to proliferate, albeit with a duplication rate reduced by two times, and they were not able to swim forward anymore but only to move slowly backward (Branche et al., 2006). Similar defects were observed in LC1RNAi mutants also deprived of outer dynein arms (Baron et al., 2007; Ralston et al., 2011).

Here, we have tested whether motility impairment could prevent trypanosomes from developing in their vector, focusing on the first crucial migration step. We found that inhibition of forward motility prevented long trypomastigote parasites from reaching the foregut and surprisingly also from differentiating into epimastigote forms. These results demonstrate for the first time in vivo that trypanosome motility has an active role in parasite migration from the tsetse midgut to the foregut and is therefore essential for the accomplishment of the parasite cycle in the tsetse fly vector.

Results

Production of DNAI1 mutant parasites

We designed a knockout strategy to replace both alleles of the DNAI1 gene by two distinct antibiotic resistance markers. Blasticidin (BLA) and neomycin (NEO-G418) resistance marker coding sequences flanked by the first 100 bp upstream of the ATG and downstream of the stop codon of DNAI1 were successively integrated into the trypanosome genome of the T. b. brucei AnTat1.1 pleomorphic strain (WT) by homologous recombination in the DNAI1 locus (Fig. 1A). These DNAI1 mutant cells (M) were subcloned by limiting dilution and several subclones were used for further experiments (M1 to M6). A pool of mutant cells was also transformed with a rescue copy of the DNAI1 gene, fused to GFP at the N-terminus end and integrated in the PFR intergenic region for a constitutive expression, to be used as an add-back control strain (AB). Correct integration of the different constructs was verified on several subclones of each strain by PCR, using primers (F2 and R2) designed to hybridize outside the first 100 bp of the DNAI1 flanking sequences used for recombination (Fig. 1A). The expected resistance markers were detected in all the different clones for each construct (Fig. 1B). In addition, a band of a size corresponding to the expected product of the endogenous locus (1968 bp) was detected in all mutant types and clones (Fig. 1B). Sequencing proved that this corresponds to an extra copy of the DNAI1 gene, and it was observed no matter the order of the transformation process (from five replicates with six subclones, not shown). Nevertheless, the intensity of this band appeared to be reduced in mutant cells compared to the untransformed WT control (Fig. 1B). At the mRNA level, a significant decrease in DNAI1 expression was observed by RT-PCR in mutant subclones compared to WT and AB trypanosomes (Fig. 1C). In agreement with the presence of an extracopy of the gene, a comparatively small amount of DNAI1 mRNA was still produced in mutant cells (Fig. 1C).

To evaluate the loss of outer dynein arms along the axoneme of these parasites at the ultrastructural level, membrane and soluble fractions of the cells were extracted by detergent treatment and the resulting cytосkeletons were analysed by transmission electron microscopy. This well-established procedure allows a better visualization of dynein arms (Branche et al., 2006). Examination of flagellum axoneme cross-sections revealed that virtually all of the nine microtubules doublets were bearing outer dynein arms in wild-type cells (Fig. 1D–E). However, one to six outer dynein arms were absent from microtubule doublets in flagellum cross-sections of DNAI1 mutant parasites (Fig. 1D–E). The correct number of outer dynein arms in AB parasites was restored, proving that the structural organization of the axoneme was recovered after the introduction of the rescue copy of the gene (Fig. 1D–E).

DNAI1 mutants are still viable despite strong motility defects

Since the expression of DNAI1 was not fully abolished in mutant cells, the resulting motility defects were scrutinized in all strains and subclones. Individual parasites were first observed under the microscope to characterize their movement. Flagella of wild-type parasites were actively beating in waves propagated most of the time from tip to base, rarely from base to tip, and resulting in forward linear movements (Movie S1). In contrast, DNAI1 mutant parasites presented uncoordinated flagellum beating
characterized by irregular base to tip waves leading to a slow rotation of the cell on itself, or chaotic cell movements of small amplitude (Movie S1). As expected, the behaviour of AB parasites in terms of flagellum beating and swimming pattern was equivalent to that of wild-type cells (Movie S1). Motility of parasite populations was then monitored by in silico tracking analyses. This also revealed strong swimming defects in DNAI1 mutants that were mostly seen tumbling, whereas WT and AB trypanosomes were moving in typical long curvilinear tracks (Fig. 2A). DNA1 mutant cells did not swim forward anymore but were occasionally seen swimming backward in the same direction. However, this was rarely sustained, the resulting motility was slower and shorter compared to WT and AB cells, and this did not reflect their principal behaviour as demonstrated in Movie S1 and Fig. 2A where only about 5% of the cells presented such a tracking pattern. Reduction of motility rapidly leads parasites to sediment at the bottom of culture flasks in liquid medium. We used this property to evidence motility impairment in mutant subclones by measuring the optical density that reflects the turbidity of the culture medium. Whereas wild-type cells were able to maintain a homogeneous distribution in the entire volume of liquid medium, DNAI1 mutant parasites sedimented rapidly (Fig. 2B). In contrast, the presence of a rescue copy of the DNAI1 gene was sufficient for the cells to remain suspended in the culture flask (Fig. 2B). DNAI1 mutants were viable and still able to proliferate, although their growth rate in culture was reduced by at least threefold compared to WT and AB cells (Fig. 2C).

In summation, although the expression of DNAI1 was not fully abolished in mutant trypanosomes, a reduction was sufficient to drastically impair motility, a phenotype...
that is fully recovered by the addition of a rescue copy of the DNAI1 gene. We took advantage of the viability of these mutants and used them for in vivo functional studies in tsetse flies.

DNAI1 mutant parasites are unable to reach the foregut of their vector

The first crucial migration step in the tsetse fly vector occurs from the posterior part of the midgut to the proventriculus. Thus, we focused our observations on the development and behaviour of the long non-dividing mesocyclic trypomastigotes involved in this migration. A total of 746 Glossina morsitans morsitans teneral males were infected by feeding with culture medium containing WT cells, DNAI1 mutant subclones 1 or 2, or AB parasites. Flies were dissected about 30 days after the infective meal (26–34 days), i.e. only after the double of the minimal time necessary to obtain a mature infection. This served to negate any possible developmental bias caused by the slower proliferation rate observed in DNAI1 mutant cells in vitro. The organs of interest were dissected independently for each fly. The posterior and anterior midgut, the proventriculus and foregut were checked for the presence of parasites under the microscope. Similar to our previous studies (Rotureau et al., 2011; 2012; Subota et al., 2011), parasites were found in 45% of the posterior midguts, 11% of the anterior midguts, and 4% of the
foreguts of flies fed with WT trypanosomes (Fig. 3A). In contrast, only 13% of posterior midguts and 7% of anterior midguts were invaded by parasites in flies fed with DNAI1 mutant parasites and no trypanosomes were detected in the foregut of these flies (Fig. 3A). These differences were all the more striking than mean infection rates observed with WT parasites were slightly lower when compared to our previous observations in the lab. This could be due to inter-experiment variations observed in this group.

Moreover, parasite densities in the different regions of the digestive tract were estimated by eyes as described previously (Subota et al., 2011) and no difference in parasite density was observed between strains in the same region (not shown). This phenotype was reversed upon expression of the DNAI1 rescue copy in AB parasites that resulted in high infection rates in the anterior midgut and foregut (Fig. 3A). These results demonstrate that DNAI1 mutants are unable to complete the first migration step of

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Fig. 3. DNAI1 mutant trypomastigotes cannot reach the proventriculus nor differentiate into epimastigotes. Flies were fed with WT trypanosomes, DNAI1 mutant subclones 1 and 2 (M1 and M2) or AB cells, and dissected from 26 to 34 days after. Whole tsetse alimentary tracts were arranged lengthways for assessment of parasite presence. PMG, posterior midgut; AMG, anterior midgut; FG, foregut; SG, salivary glands.

A. Infection rates were plotted as mean ± SD according to the region of the digestive tract. Total numbers of dissected flies from at least three separate experiments are indicated. One-way ANOVA tests, with intergroup comparisons by Tukey ad hoc post-tests with $\alpha = 0.05$, were performed and significant results were indicated with * $P < 0.05$. B. Parasites extracted from the AMG of infected flies were fixed in methanol and stained with DAPI. Arrows indicate epimastigote forms in representative fields for WT and AB infections. Scale bar represents 10 μm. C. Parasites were typed according to their morphology and DNA staining pattern (relative positions of the kinetoplasts and nuclei). Cell populations were quantified and plotted by stage as percentage of the total number of parasites for each strain. Total numbers of cells randomly observed from at least three distinct experiments are given for each strain. PC: procyclic trypomastigote; MS: mesocyclic trypomastigote; DE: long dividing epimastigote; LE: long epimastigote; SE: short epimastigote.
their parasite cycle in the tsetse fly from the posterior midgut to the foregut, and that this phenotype is related to the expression of DNAI1.

**DNAI1 mutant trypanmastigotes are unable to differentiate into epimastigotes**

When living parasites extracted from the midgut of infected flies were examined under the microscope, DNAI1 mutant parasites presented uncoordinated flagellum beating characterized by irregular base to tip waves that led to chaotic cell movements, whereas WT and AB trypanosomes were actively beating their flagellum in waves propagated most of the time from tip to base. This resulted in efficient forward linear movements only in the WT and AB strains (Movie S2). These observations confirm that the DNAI1 mutant motility phenotype was maintained in midgut trypanmastigotes, and demonstrate that despite this impairment some DNAI1 mutant parasites could yet progress in the direction of the proventriculus.

The most striking difference between the DNAI1 mutants and the WT and AB cells was observed when the cells extracted from the anterior midgut were typed according to their general morphology and positions of their nucleus and kinetoplast in relation to one another along the anterior-posterior axis of the cell (Fig. 3B). The quantification of cell population by stage revealed that whereas 19% of control parasites had differentiated into epimastigotes, all DNAI1 mutant trypanosomes remained in the trypanmastigote morphotype (Fig. 3B–C). Here too, this phenotype was complemented in parasites bearing a rescue copy of the DNAI1 gene, among which 7% of trypanosomes were in the epimastigote stage (Fig. 3B–C).

**Discussion**

Motility was proposed to play a crucial role during the early development of trypanosomes in the tsetse fly, especially during their first migration step from the posterior midgut to the foregut (Van Den Abbeele et al., 1999). Trypanosome migration could be passive as it is the case for *Plasmodium* sporozoites bathing in the *Anopheles* haemolymph that passively reach the salivary glands after their egress from the oocytes (Hillyer et al., 2007). However, there is no evidence for the presence of trypanosomes in the tsetse haemolymph, and it seems unlikely that 30 μm long trypanosomes could passively diffuse along a digestive tract more than 5 cm in length, against the active forces of digestive flow and peristalsis, and in a highly viscous environment. Thus, trypanosome migration likely relies on active and directional motion.

Trypanosome motility has been extensively analysed from culture observations (Ralston et al., 2009). If the trypanosome flagellum can produce bidirectional waves, tip to base beating is observed most of the time in liquid medium, and this results in the cell traction (Branche et al., 2006). However, the natural environments of trypanosomes are not as liquid as the commonly used synthetic culture media. Consequently, despite important technical issues, analyses of trypanosome motion in their natural environments are now required.

*Trypanosoma brucei* variant surface glycoproteins form a dense surface coat involved in antigenic variation. Their sorting by hydrodynamic forces is thought to help trypanosomes to protect against complement-mediated immune destruction (Engstler et al., 2007). One could propose that cell orientation defects could render this mechanism inefficient. Nevertheless, the importance of forward swimming *in vivo* is far from a consensus, mostly because of the controversial RNAi phenotypes observed so far that are systematically lethal regardless of the targeted flagellum protein (Hill, 2010). In contrast, little or no growth deficit has been reported in motility-deficient procyclic trypanosomes in culture such as in DNAI1^RNAi* (Branche et al., 2006), PFR2^RNAi* (Bastin et al., 1998), Trypanin^RNAi* (Hutchings et al., 2002) or LC1^RNAi* mutants (Baron et al., 2007; Ralston et al., 2011). In agreement with these observations of monomorphic strains *in vitro*, the pleomorphic DNAI1 mutant parasites were still able to invade the posterior midgut and to proliferate. Moreover, despite having motility that is strongly impaired, they were able to reach the anterior midgut of the fly. Protozoan motions in low Reynolds number environments are not fully understood. In addition, the nature and physical properties of the viscous microenvironments imposed on trypanosomes in the tsetse midgut are likely to be frequently modified with the digestion process. This could influence parasite motion efficiency in a way that would favour, or at least not disfavour, the backward movements of DNAI1 mutants. Another, yet more unlikely, explanation is a possible cooperation between cells that could synchronize their movements or promote cooperation (Oberholzer et al., 2010). This would result in a progressive movement of DNAI1 mutants in the direction of the tsetse proventriculus. However, this migration was limited to the anterior region of the midgut in DNAI1 mutant trypanastigotes, suggesting that the conditions for parasite migration after this point could be different. From this region of the digestive tract, forward motility appeared to be crucial for parasite migration. Again, it could be hypothesized that the nature of the microenvironment (e.g. lower viscosity around the proventriculus), and/or the reduction in parasites density (Oberle et al., 2010).
An unexpected modification in parasite development emerged in the DNAI1 mutant strain: the absence of epimastigote forms in the anterior midgut. Although the link between flagellum beating and the molecular processes involved in cytoskeleton remodelling and nucleus repositioning remain unclear (Sharma et al., 2008; Rotureau et al., 2011), this could reflect for the first time a direct involvement of motility in cell differentiation. As the parasite flagellum was proposed to act as a sensing antenna that could scan the micro-environment at the front of the cell (Oberholzer et al., 2011; I. Subota et al., unpubl. data), it could be proposed that an incorrect orientation of the flagellar tip during the migration, or the preponderance of base to tip waves, could prevent the proper reception of external stimuli and/or the subsequent intracellular signalling of information leading to the initiation of differentiation.

Our difficulties to fully knockout DNAI1 genes due to the presence of an extra copy nonetheless allowed us to produce mutant parasites with strong motility defects but yet viable. This reinforces the importance of the flagellar outer dynein arms in trypanosome biology as the DNAI1 mutant mesocyclic parasites were neither able to reach the foregut nor able to differentiate into epimastigote forms, two key steps of parasite development. The complementation of the phenotypes observed in DNAI1 mutants by the addition of a rescue copy of the DNAI1 gene confirms that both the absence of forward motility and the inability to differentiate into epimastigotes are specifically due to the reduction of the amount of DNAI1 in mutant parasites. In other words, forward motility is essential for the long mesocyclic trypanomatid parasites to assume their function of invading the tsetse foregut and paving the way for the parasite population to ultimately infect to tsetse salivary glands. In summation, this is the first demonstration that trypanosome swimming is essential for the completion of the parasite cycle in vivo. The fact that motility impairment could break the parasite cycle opens new approaches for the control of diseases caused by trypanosomatids.

Experimental procedures

Trypanosome cell lines and cultures

All vectors were separately nucleofected (Lonza) in the pleomorphic strain T. brucei brucei AnTat1.1 (Le Ray et al., 1977) at the freshly differentiated procyclic stage (≤2 weeks). Procylic trypanosomes were maintained in SDM79 medium (Brun and Schonenberger, 1979) supplemented with 10% fetal bovine serum and 20 mM glycerol. Antibiotic-resistant cells with the most pronounced motility phenotype or the highest EGFP signal were selected for subcloning by limiting dilution and named M1 to M6. Cell culture growth was monitored daily with a Z2 cell counter (Beckman Coulter).

Generation of DNAI1 mutant and AB cell lines

Two alleles of the DNAI1 gene (Tb11.02.2640) were successively replaced by two distinct antibiotic resistance cassettes. Constructs composed of the full BLA (399 bp) and NEO (867 bp) coding sequences flanked by the first 100 bp upstream the start codon and downstream the stop codon of the DNAI1 coding sequence were chemically synthesized (GeneCust Europe). Inserts were amplified with the primers F1 and R1 selected to hybridize to the 100 bp region immediately flanking the DNAI1 coding sequence that were used for recombination of the two resistance markers: CGAAGCAACCGTAGAGGACTCCGTAGT as forward primer F1 and TTGCCTCATTCAAATCCCATCACAAAC as reverse primer R1. Inserts were integrated in the trypanosome genome via homologous recombination in the DNAI1 locus via two successive rounds of nucleofection (Lonza). Subclones of double mutant cells were transformed with a rescue copy of the DNAI1 gene tagged to EGFP and integrated in the PFR intergenic region of the trypanosome nuclear genome to be used as an add-back control strain (AB). For this purpose, the full DNAI1 coding sequence (1968 bp) was cloned with Nhel and EcoRI at the 3′ end of the EGFP coding sequence in the pPCFPR-EGFP vector (Absalon et al., 2008; Adhiambo et al., 2009).

Tsetse fly infection, maintenance and dissection

Teneral males of G. morsitans morsitans from 24 to 96 h post-eclosion were obtained from the UMR177 IRD-CIRAD, Campus International de Baillarguet, Montpellier, France. Tsetse flies were allowed to ingest parasites in culture medium during their first meal through a silicone membrane. Cultured procyclic trypanosomes were used at 5 × 10^6 cells per ml in SDM79 medium supplemented with 10% fetal bovine serum, 60 mM N-acetylgalacosamine (Peacock et al., 2006) and 2.5% (w/v) bovine serum albumin (Kabayo et al., 1986). Tsetse flies were subsequently maintained in Roubaud cages at 27°C and 70% humidity and fed twice a week through a silicone membrane with fresh defibrinated sheep blood.

Flies were starved for at least 48 h before being dissected from 26 to 34 days after ingestion of the infected meal. Whole tsetse alimentary tracts, from the distal part of the foregut to the rectum, were then dissected and arranged lengthways for assessment of parasite presence. Foregut and proventriculus were physically separated from the midgut in distinct PBS drops. Tissues were dilacerated and recovered parasites were treated for further experiments no more than 15 min after dissection.

Motility analyses

For motility analysis, two different tests were performed in three separate experiments: sedimentation assay and in silico tracking. Interfering with flagellum motility reduces cell motility resulting in cell sedimentation at the bottom of the culture flask. This can be monitored in sedimentation assays by measuring the optical density (OD) of cultures as previously described (Bastin et al., 1999; Branche et al., 2006). In addition, the 2D tracks characterizing cell motility were obtained by in silico tracking experiments. For each strain, 10–20 movies were recorded (200 frames, 50 ms of exposure). Samples were observed in culture medium
maintained at 27°C at 5 × 10⁶ cells ml⁻¹ under the 10× objective of an inverted DMI-4000B microscope (Leica) coupled to a Retiga-SRV camera (QImaging). Movies were converted with the MPEG Streamclip V.1.9b3 software (Squared 5) and analysed with the medeaLAB CASA Tracking V.5.5 software (medea AV GmbH). For a more precise observation, movies of individual cells (200 frames, 100 ms of exposure) were also recorded. Samples from cultures or dissected organs were observed under cells (200 frames, 100 ms of exposure) were also recorded. Movies were compiled in iMovie 9.0.4 (Apple).

Immunofluorescence

For immunofluorescence, parasites extracted from the anterior midgut were settled on poly-l-lysine coated slides and fixed in methanol at −20°C for at least 5 min and re-hydrated in PBS for 10 min. Slides were stained with 4',6-diamidino-2-phenylindole (DAPI) for visualization of kinetoplast and nuclear DNA content, washed and mounted under coverslips with ProLong antifade reagent (Invitrogen). Samples were observed either (i) with a DMR microscope (Leica) and images were captured with a CoolSnap HQ camera (Roper Scientific), or (ii) with a DMI4000 microscope (Leica) and images were acquired with a Retiga-SRV camera (Q-Imaing).

Electron microscopy

For preparation of cytoskeletons, cells were treated with 1% Nonidet P-40 at 4°C in PBS for 10 min to strip the plasma membrane. Samples were washed twice in PBS, fixed, embedded and sectioned for transmission electron microscopy as described previously (Branche et al., 2006).

PCR and RT-PCR

Genomic DNA was extracted from each strain and purified using phenol/chloroform (Rotureau et al., 2005). PCR was performed according to the manufacturer recommendations with a GoTaq kit (Promega). Primers F2 and R2 were selected to hybridize 200 bp upstream of the start codon and 200 bp downstream of the stop codon of the DNAI1 coding sequence, in order to verify the correct insertions of the drug markers (Fig. 1A): CCATCCTTC AAGTACACCATCAAT as forward primer F2 and GAGGATATAT ACACACACATTTA as a reverse primer R2.

Total RNAs were extracted from each strain and purified using TRIzol (Invitrogen). DNA was eliminated by DNase treatment (Qiagen) and RNA purity was confirmed by conventional PCR. After primer calibration and determination of optimal conditions, semi-quantitative RT-PCR was performed according to the manufacturer recommendations with a SuperScript One-step RT-PCR Platinum-Taq kit (Invitrogen). Primers were selected to amplify short regions in the coding sequences of the following genes: GAATCCGTTCCATCCCAGTGTC (forward) and TCA CGCTGGGCCAGGA (reverse) targeting a 470 bp sequence of DNAI1, and ATGGTGGCGAGACT (forward) and GCCCGACG GTTTTGTC (reverse) targeting the first 105 bp of the FLA1 gene as control. In parallel to RT-PCR targeting DNAI1, the same samples were processed for RT-PCR targeting FLA1 and for direct PCR targeting DNAI1 as positive and negative controls respectively. Internal negative controls with H₂O for RT-PCR and positive control with WT genomic DNA for PCR were also performed.

Statistical analyses

Statistical analyses were performed in Excel or with the KaleidaGraph V.4.0 software (Synergy Software). Infection rates were plotted as mean ± SD. One-way ANOVA tests, with inter-group comparisons by Tukey ad hoc post-tests with α = 0.05, were performed and significant results were indicated with *P < 0.05 (Fig. 3A).

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References

Absalon, S., Blisnick, T., Kohl, L., Toutirais, G., Dore, G., Julkowska, D., et al. (2008) Intraflagellar transport and functional analysis of genes required for flagellum formation in trypanosomes. Mol Biol Cell 19: 929–944.

Adhiambo, C., Blisnick, T., Toutirais, G., Delannoy, E., and Bastin, P. (2009) A novel function for the atypical small G protein Rab-like 5 in the assembly of the trypanosome flagellum. J Cell Sci 122: 834–841.

Baron, D.M., Kabututu, Z.P., and Hill, K.L. (2007) Stuck in reverse: loss of LC1 in Trypanosoma brucei disrupts outer dynein arms and leads to reverse flagellar beat and backward movement. J Cell Sci 120: 1513–1520.

Bastin, P., Sherwin, T., and Gull, K. (1998) Paraflagellar rod is vital for trypanosome motility. Nature 391: 548.

Bastin, P., Pullen, T.J., Sherwin, T., and Gull, K. (1999) Protein transport and flagellum assembly dynamics revealed by analysis of the paralysed trypanosome mutant snl-1. J Cell Sci 112 (Part 21): 3769–3777.

Branche, C., Kohl, L., Toutirais, G., Buisson, J., Cosson, J., and Bastin, P. (2006) Conserved and specific functions of axoneme components in trypanosome motility. J Cell Sci 119: 3443–3455.

Brun, R., and Schonenberger. (1979) Cultivation and in vitro cloning or procyclic culture forms of Trypanosoma brucei in a semi-defined medium. Short communication. Acta Trop 36: 289–292.

Brun, R., Blum, J., Chappuis, F., and Burri, C. (2009) Human African trypanosomiasis. Lancet 375: 148–159.
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Chappuis, F., Lima, M.A., Flevaud, L., and Ritmeijer, K. (2010) Human African trypanosomiasis in areas without surveillance. Emerg Infect Dis 16: 354–356.

Engstler, M., Pfohl, T., Herminghaus, S., Boshart, M., Wiegerjtes, G., Heddergott, N., and Overath, P. (2007) Hydrodynamic flow-mediated protein sorting on the cell surface of trypanosomes. Cell 131: 505–515.

Griffiths, S., Portman, N., Taylor, P.R., Gordon, S., Ginger, Hillyer, J.F., Barreau, C., and Vernick, K.D. (2007) Efficiency of gliding locomotion in eukaryotes. Int Rev Cytol 251: 79–129.

Hutchings, N.R., Donelson, J.E., and Hill, K.L. (2002) Trypanosoma brucei and Hill, K.L. (2009) The Trypanosoma brucei flagellum: moving parasites in new directions. Annu Rev Microbiol 63: 335–362.

Ralston, K.S., Kisalu, N.K., and Hill, K.L. (2011) Structure-function analysis of dynein light chain 1 identifies viable motility mutants in bloodstream-form Trypanosoma brucei. Eukaryot Cell 10: 884–894.

Robinson, D.R., and Gull, K. (1991) Basal body movements as a mechanism for mitochondrial genome segregation in the trypanosome cell cycle. Nature 352: 731–733.

Rotureau, B., Gego, A., and Carme, B. (2005) Trypanosomad protozoa: a simplified DNA isolation proce- dure. Exp Parasitol 111: 207–209.

Rotureau, B., Subota, I., and Bastin, P. (2011) Molecular bases of cytoskeleton plasticity during the Trypanosoma brucei parasite cycle. Cell Microbiol 13: 705–716.

Sharma, R., Peacock, L., Gluenz, E., Gull, K., Gibson, W., and Carrington, M. (2008) Asymmetric cell division as a route to reduction in cell length and change in cell morphology in trypanosomes. Protist 159: 137–151.

Sharma, R., Gluenz, E., Peacock, L., Gibson, W., Gull, K., and Carrington, M. (2009) The heart of darkness: growth and form of Trypanosoma brucei in the tsetse fly. Trends Parasitol 25: 517–524.

Simarro, P.P., Jannin, J., and Cattand, P. (2008) Eliminating human African trypanosomiasis: where do we stand and what comes next? PLoS Med 5: e55.

Subota, I., Rotureau, B., Blisnick, T., Ngwabyt, S., Durand-Dubief, M., Engstler, M., and Bastin, P. (2011) ALBA proteins are stage regulated during trypanosome development in the tsetse fly and participate in differentia- tion. Mol Biol Cell 22: 4205–4219.

Tavares, P., Sansonetti, P., and Guillen, N. (2000) Cell polarization and adhesion in a motile pathogenic protozoan: role and fate of the Entamoeba histolytica Gal/GalNAc lectin. Microbes Infect 2: 643–649.

Van Den Abbeele, J., Claes, Y., van Bockstaele, D., Le Ray, D., and Coosemans, M. (1999) Trypanosoma brucei spp. development in the tsetse fly: characterization of the post-mesocyclic stages in the foregut and proboscis. Parasitology 118: 469–478.

Vickerman, K. (1985) Developmental cycles and biology of pathogenic trypanosomes. Br Med Bull 41: 105–114.

Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher’s web-site:

Movie S1. DNA11 mutant motility defects in vitro. Movies of individual WT, DNA11 mutant M2 and AB trypanosomes observed in liquid culture medium under a 100× objective.

Movie S2. DNA11 mutant motility defects in vivo. Movies of trypanosomes extracted from the anterior midgut of tsetse flies fed 26 days before with WT, DNA11 mutant M2 and AB trypanosomes and observed under a 40× objective.