 Trafficking activity of myosin XXI is required in assembly of *Leishmania* flagellum

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Summary

Actin-based myosin motors have a pivotal role in intracellular trafficking in eukaryotic cells. The parasitic protozoan organism *Leishmania* expresses a novel class of myosin, myosin XXI (Myo21), which is preferentially localized at the proximal region of the flagellum. However, its function in this organism remains largely unknown. Here, we show that Myo21 interacts with actin, and its expression is dependent of the growth stage. We further reveal that depletion of Myo21 levels results in impairment of the flagellar assembly and intracellular trafficking. These defects are, however, reversed by episomal complementation. Additionally, it is shown that deletion of the *Myo21* gene leads to generation of ploidy, suggesting an essential role of Myo21 in survival of *Leishmania* cells. Together, these results indicate that actin-dependent trafficking activity of Myo21 is essentially required during assembly of the *Leishmania* flagellum.

Key words: Trypanosomatids, Intraflagellar transport, Myosin, Trafficking

Introduction

Myosin proteins constitute a group of actin-based molecular motors that produce movement along the actin filaments and have diverse motile functions in eukaryotic cells, ranging from muscle contraction and pseudopodial motility, to vesicle transport and cytokinesis (Woolner and Bement, 2009). These proteins share a common domain plan, consisting of N-terminal head domain (motor domain) responsible for actin binding and ATPase activity, a light-chain-binding neck domain, and a C-terminal tail domain that imparts functional specificity to different classes of myosins (Krendel and Mooseler, 2005). Owing to a high degree of sequence conservation in the head domain, myosin has been anticipated to power their movements along F-actin tracks, whereas divergent tail domains bind a diverse array of partners, including proteins and membranes (Karcher et al., 2002). Based on variations in the amino-acid sequence and domain composition, myosins have been classified into more than 30 classes in different organisms (Odrontitz and Kollmar, 2007; Foth et al., 2006). Genomes of kinetoplastid parasites, such as *Trypanosoma* and *Leishmania*, encode a class of myosins (El-Sayeed et al., 2005) that were classified as myosin XXI (Myo21) by Foth and co-workers (Foth et al., 2006). Myo21, unlike other myosin proteins, contains UBA-like protein domains and has no structural or functional relationship with the myosins present in other organisms possessing cilia or flagella (Odrontitz and Kollmar, 2007; Foth et al., 2006).

*Leishmania* are a group of trypanosomatid parasites that cause a number of life-threatening human diseases including ‘Kala-azar’ (Desjeux, 2004). These parasites mainly exist in two forms: (1) the non-motile amastigote, residing within the mammalian macrophages, and (2) highly motile promastigote, which reside in the alimentary tract of the sandfly vector. The amastigote forms possess only rudimentary flagella, whereas the promastigote forms actively swim using their single long flagellum. The basic scaffold of the eukaryotic flagellum consists of nine characteristically arranged outer microtubule doublets encircling two central-pair microtubules that are interlinked with several discrete substructures, such as radial spokes, inner and outer dynein arms, nexins and other intricacies ascribing specific flagellar functions. This structure in trypanosomatids, including *Leishmania*, shows an increased complexity, because as well as the axoneme, it also contains an extra-axonemal protein lattice known as the paragflagellar rod (PFR). The PFR runs along the entire length of the flagellum and is crucial for cell motility in trypanosomatids (Santrich et al., 1997).

In *Trypanosoma*, yet another subflagellar structure has been described: a transmembrane mobile junction, known as a flagella connector (FC). This structure connects the tip of the growing flagellum with the old one during cell division (Moreira-Leite et al., 2001; Briggs et al., 2004). The flagellum in these organisms is attached to the cell body through the basal body, and its membrane is continuous with a cup-shaped membrane invagination, called the flagellar pocket (Field and Carrington, 2009).

The flagellum, in general, is a dynamic microtubule-based cellular protrusion that imparts motility and/or sensory functions associated with a range of biological processes related to human health. The dynamics of flagella involve a process of assembly and disassembly, the knowledge of which is now beginning to emerge. This process requires movements of protein cargoes from the flagellum base to its tip (anterograde) and also from the tip to its base (retrograde) and is termed as ‘intraflagellar transport’ (IFT) (Rosenbaum and Witman, 2002). Based on the existing knowledge, IFT is powered by the microtubule-based motor proteins such as kinesin II and dynein complexes for anterograde and retrograde transports, respectively (Cole and Snell, 2009). However, the flagella of trypanosomes and *Leishmania* as well as containing microtubule-based axonemal structures also contain PFRs, but the mechanism by which the PFR proteins are transported and
assembled within the flagellar compartment remains unknown. Furthermore, the FC (flagella connector) has been shown to move beyond the axoneme, even in the absence of IFT (Kohl et al., 2003; Davidge et al., 2006). It thus appears that the existing knowledge of the transport of proteins into and within the flagellar compartment is still incomplete.

In addition to microtubules and microtubule-based motor proteins, the flagellar compartment also contains actin, actin-related proteins and actin-binding proteins in a variety of organisms (Muto et al., 1994; Yanagisawa and Kamiya, 2001; Erfeld and Gull, 2001; Sahasrabuddhe et al., 2004; Minoura, 2005; Broadhead et al., 2006). However, little is known about the functions of the actin-based cytoskeletal elements in the flagellum. In flagella of Chlamydomonas, actin has been reported to form an important subunit of the inner-arm dynein (Kato-Minoura et al., 1997; Hayashi et al., 2001). In addition, the actin-dynamics-regulating protein ADF/cofilin (Cof), has recently been shown to be involved in assembly of the Leishmania flagellum (Tammana et al., 2008), suggesting a role of actin dynamics in this process. As the Leishmania flagellum, besides containing actin and Cof (Sahasrabuddhe et al., 2004; Tammana et al., 2008) also contains Myo21 (Katta et al., 2009), we speculate that actomyosin-based transport of flagellar proteins might also be required during flagellum assembly. To test this possibility, we analyzed the effects of Myo21 gene deletion on flagellum assembly and intracellular transport in Leishmania cells. Our results show that Myo21 is essentially required in both flagellar assembly and cell survival.

Results

Myo21 associates with actin in Leishmania cells

The Leishmania genome encodes two myosin genes: one that corresponds to class I myosin, whereas the other is a novel class of myosin, myosin XXI (Myo21) (Foth et al., 2006). However, our recent studies have shown that Leishmania express only Myo21, which, in the promastigotes is largely concentrated in the proximal region of the flagellum, with a diffuse distribution in other flagellar and cell-body compartments (Katta et al., 2009). This differential Myo21 distribution has now been confirmed by comparing the localization of Myo21 in Leishmania promastigotes before and after NP-40 treatment (supplementary material Fig. S1). As this protein has also been reported to partially colocalize with actin in Leishmania cells (Katta et al., 2009), we reanalyzed the Myo21 association with actin using immunofluorescence microscopy and co-immunoprecipitation techniques.

As myosins interact only with the filamentous form of actin, which is infrequently seen in Leishmania cells, compared with eukaryotic cells (Nayak et al., 2005), we mainly focused on the cells that clearly showed actin filaments or patches. Myo21 colocalized with actin in such cells (Fig. 1A; also see supplementary material Fig. S1) although the extent of Myo21 signal varied from cell to cell. To analyze whether actin interacts with the head domain or tail domain of Myo21, the promastigotes that expressed GFP conjugates of H-Myo21 (head domain) and T-Myo21 (tail domain) along with full length Myo21 (Katta et al., 2009) were used in immunoprecipitation experiments (Fig. 1B). The cells were first lysed with Triton X-100 (1% v/v) and clear detergent-soluble fractions were subjected to immunoprecipitation using anti-GFP antibodies. Analysis of the immunoprecipitates revealed that actin co-immunoprecipitated with full-length Myo21 and H-Myo21, but not with T-Myo21 (Fig. 1C). These results not only confirmed the existence of acto-Myo21 complexes in Leishmania promastigotes but also revealed that the head domain of Myo21 was responsible for its interactions with actin.

Myo21 expression is growth specific and correlates with flagellar length

Flagellar length in Leishmania is closely linked to the cell life cycle and growth phase (Cuvillier et al., 2003; Zakai et al., 1998), with flagella twice the length of the cell body in metacyclic promastigotes and a short rudimentary flagellum in amastigotes (Rogers et al., 2002). As Myo21 expression depends on the parasite life cycle (Katta et al., 2009), we also examined whether this expression varied with the parasite growth phase. Interestingly, Myo21 expression levels increased gradually up to ~4.5-fold with the lowest in the early-log phase and the highest in the stationary phase (Fig. 2A). This was confirmed by measuring mRNA levels using semi-quantitative RT-PCR (supplementary material Fig. S2). This difference in the Myo21 expression levels between the different growth phases was also apparent in immunofluorescence
Myo21 is essentially required for survival of *Leishmania* cells

To investigate the role of Myo21 in assembly of *Leishmania* flagellum as well as in other cellular processes, we knocked out the Myo21 gene in *L. donovani* promastigotes. Genome sequence data of *L. major*, *L. infantum* and *L. braziliensis* have revealed single-copy architecture of the Myo21 gene in their genomes (www.genedb.org), which we also confirmed in our *L. donovani* strain (supplementary material Fig. S3). As it is a diploid organism, *L. donovani* carries two myosin alleles. To replace both these alleles, two gene deletion constructs were prepared with different antibiotic-resistance markers: neomycin phosphotransferase (*Neo*) and hygromycin phosphotransferase (*Hyg*) genes, which confer resistance to the antibiotics G-418 and hygromycin B, respectively. Deletion constructs contained 950 bp upstream and 1200 bp downstream regions of the *Myo21* gene at the 5’ and 3’ ends of the antibiotic-resistance genes, respectively (Fig. 3A).

Both the *Neo* and *Hyg* cassettes were separately transfected in *L. donovani* promastigotes by electroporation and antibiotic-resistant clones were selected on DMEM-agar plates containing G-418 or hygromycin B (10 µg/ml each). These clones were then analyzed for the replacement of *Myo21* allele by Southern blotting using *Neo* or *Hyg* gene probes. All the colonies screened (more than 50) from both the G-418 and hygromycin plates were found to have integration of deletion cassettes at the *Myo21* gene locus and Southern blots of one clone each from G-418- and hygromycin-resistant colonies are shown (Fig. 3B). Western blot analysis showed approximately 40-50% depletion of *Myo21* levels in these clones (Fig. 3C). To obtain a *Myo21*-null mutant, the hygromycin resistant heterozygous clone was subjected to a second round of transfection with the neomycin construct. Clones selected on the DMEM-agar plates containing G-418 and hygromycin B (10 µg/ml each) were subsequently analyzed by both Southern and western blotting. To our surprise, despite replacement of both the alleles by *Hyg* and *Neo* genes (a total of 100 colonies screened), *Myo21*-null mutants could not be obtained (Fig. 3B,C, also see supplementary material Fig. S4). Expression levels of *Myo21* in double allele-replaced mutants (*Myo21*+/−) were however comparable with the heterozygous clones having only single allele replacement (Fig. 3C), and was consistent in five randomly selected *Myo21*−/− clones (supplementary material Fig. S4). Furthermore, flow-cytometric analysis showed no gross change in the DNA content of the *Myo21*−/− clones compared with wild-type and *Myo21*+/− cells (supplementary material Fig. S4), suggesting generation of aneuploidy in the *Myo21*−/− clones. As ploidy generation in *Leishmania* has been viewed as a measure of essentiality of genes (Mottaram et al., 1996; Cruz et al., 1993; Dumas et al., 1997; Tovar et al., 1998), *Myo21* expression can be regarded essential for survival of the *Leishmania* cells in culture. In the absence of *Myo21*+/− mutants, we episomally expressed *Myo21*-GFP (Katta et al., 2009) in one of the hygromycin-B-resistant heterozygous clones (*Myo21*+/−) for add-back analyses (Fig. 3D). The *Myo21*+/− cells...
Fig. 3. Deletion of Myo21 gene. (A) Schematic representation of Myo21 locus in *L. donovani* showing size differences before and after integration of Neo or Hyg cassettes after restriction digestion by Apal. (B) Southern blot analysis of the Myo21+/+ and Myo21+/– mutants confirming replacement of Myo21 alleles. Signal of Myo21 in Myo21+/+ homozygous mutant indicates ploidy generation. EthBr, ethidium bromide; Myo, Hyg and Neo, southern blots probed with Myo21, Hyg and Neo gene probes, respectively. (C) Western blot analysis of wild-type (Myo21+/+), Myo21+/– Neo and Myo21+/– cells in the mid-log phase showing depletion of Myo21 in heterozygous as well as in homozygous mutants. a, Coomassie-Blue-stained PVDF membrane (40 μg protein per lane); b, western blot probed with anti-Myo21 and anti-actin antibodies. (D) Expression analysis of Myo21-GFP in Myo21+/–Comp cells in the mid-log phase. a, Ponceau-stained nitrocellulose membrane (40 μg protein per lane); b, western blot probed with anti-Myo21 and anti-actin antibodies. Lane 1, wild type; lane 2, Myo21+/–; lane 3, Myo21+/–Comp cells (triangles). Calculated generation time (in hours) for wild type, 10.40±0.45; Myo21+/– cells, 13.05±0.70; Myo21+/–Comp, 11.20±0.5. +/–, wild-type cells; +/–Neo, neomycin-resistant heterozygous Myo21 mutant; +/–Hyg, hygromycin-resistant heterozygous Myo21 mutant; +/–Comp, neomycin-resistant homozygous Myo21 mutant.

Fig. 4. Analysis of cell shape, flagellar length, motility and Myo21 expression in Myo21 mutants. (A) Scanning electron microscopy images showing stumpy cell body and reduced flagellar length of Myo21+/– (+/–) cells, which reverted back to normal in Myo21+/–Comp (+/–Comp) cells. Scale bars: 5 μm. (B) Histograms of flagellar length of wild-type (+/+), Myo21+/– (+/–) and Myo21+/–Comp (+/–Comp) cells. More than 200 cells were analyzed from each cell line. (C) Motility analysis of Myo21 mutants by time-lapse microscopy. Traced paths of live, individual cells from a time-lapse movie of 36 seconds, showing non-motile nature of Myo21+/– (+/–) cells compared with wild-type (+/+) cells and attainment of normal motility in Myo21+/–Comp (+/–Comp) cells. Scale bars: 100 μm. (D) Western blot analysis of the wild type (+/+), Myo21+/– (+/–) and Myo21+/–Comp (+/–Comp) cells showing the absence of growth-dependent increase in Myo21 levels in Myo21+/– and Myo21+/–Comp cells (40 μg protein per lane). Actin, internal loading control; *, Myo21-GFP; e-log, early-log-phase cells; m-log, mid-log-phase cells; stat, stationary-phase cells.

exhibited slightly reduced growth, which was restored to normal upon episomal complementation with Myo21-GFP (Myo21+/–Comp) (Fig. 3E). However, H-Myo21-GFP or T-Myo21-GFP failed to complement Myo21+/– cells, suggesting that both the head and the tail domains are essentially required for Myo21 function.

**Reduction in Myo21 levels results in reduced flagellar length and absence of PFRs**

Both the single and double mutants appeared to have similar expression levels of Myo21. Therefore, one of the hygromycin-B-resistant heterozygous clones (Myo21+/–) was used for detailed analyses throughout the present study and wherever necessary, more clones were included for confirmation of the results. Since Myo21 expression levels varied with cell growth, all the analyses were done strictly using ‘mid-log-phase’ cells (supplementary material Fig. S5), unless specifically stated otherwise. Overall, the heterozygous mutants appeared short and stumpy (Fig. 4A), and possessed significantly reduced flagellar lengths averaging 2.13±1.65 μm (n=200), compared with 12.40±4.97 μm (n=204) in the wild-type cells (Fig. 4B; also see supplementary material Fig. S6). The short flagella in Myo21+/– cells exhibited occasional bending movements which were not enough to propel the cell forward (Fig. 4C; also see supplementary material Movie 1). Since wild-type cells exhibited variable expression of Myo21 at different stages, it was essential to examine whether the same was true for Myo21+/– cells. However, no significant change in the flagellar length with the growth stage was observed in the mutant cells (Fig. 4D). Myo21 expression also remained unchanged in different growth phases of Myo21+/–Comp cells. These results further confirm that Myo21 expression is not directly involved in growth-phase-specific regulation of flagellar length.
Immunofluorescence analysis of Myo21<sup>+/–</sup> cells showed the presence of Myo21, with a dot-like appearance at the proximal region of the rudimentary flagellum (supplementary material Fig. S7A). To examine whether relative distribution of Myo21 in NP-40-insoluble and -soluble fractions was altered in Myo21<sup>+/–</sup> cells, compared with wild-type cells, we analyzed these fractions by western blotting; no difference in the relative distributions of Myo21 was observed between the mutants and wild-type cells (supplementary material Fig. S7B).

The trypanosomatid flagellum is marked by an additional structure called the PFR (Bastin et al., 1996). We, therefore, examined the expression and location of PFR proteins in Myo21<sup>+/–</sup> cells using mAb2E10 antibodies, which detect major PFR proteins, i.e. PFR1 and PFR2, in the flagellum of Leishmania promastigotes (Ismach et al., 1989). Unlike wild-type Leishmania promastigotes, no PFR staining was seen in Myo21<sup>+/–</sup> cells (Fig. 5A). Further analysis revealed that in contrast to the wild-type cells, expression levels of the PFR proteins were undetectable in Myo21<sup>+/–</sup> cells, which were restored to normal by episomal complementation (Fig. 5B). Transmission electron microscopy revealed that the PFR structure was altogether absent in the flagella of Myo21<sup>+/–</sup> cells (Fig. 5C). Nevertheless, the canonical 9+2 arrangement of the axoneme microtubules appeared intact and no accumulation of vesicles or electron-dense particles could be seen in the flagellar ultrastructure of Myo21<sup>+/–</sup> cells. Importantly, the PFR was regained after episomal complementation with Myo21-GFP. These results suggest that Myo21 is essentially required in flagellar assembly and elongation in Leishmania.

**Myo21<sup>+/–</sup> cells are defective in intracellular trafficking**

Transmission electron microscopy analyses of about 90% of cross-sections of Myo21<sup>+/–</sup> cells revealed that the flagellar pockets were 3-4 times larger (750-1200 nm diameter, n=62, 57/62) compared with wild-type cells (280-340 nm diameter, n=59) (Fig. 6), suggesting a defect in vesicular transport (Garcia-Salcedo et al., 2004; Allen et al., 2003) in these cells. To confirm this observation, we analyzed the transport of endocytic vesicles in Leishmania promastigotes by using the fluorophore N-(3-triethylammoniumpropyl)-4-[6-[4-(diethylamino)phenyl]-hexatrienyl]pyridinium dibromide (FM4-64), which has been widely used as a marker for assessing the flagellar-pocket activity in trypanosomatids (Sahin et al., 2008; Mullin et al., 2001). In wild-type cells, flagellar pockets were typically marked at 4°C, and the fluorophore efficiently endocytosed and trafficked down to form a characteristic multivesicular tubule (Ghedin et al., 2001) at 25°C within 1 hour (Fig. 7A). By contrast, in about 56% of Myo21<sup>+/–</sup> cells (n=660, 294/660), the fluorophore remained associated with the flagellar pocket and failed to get endocytosed under identical conditions. These results were verified independently in a total of four randomly selected heterozygous clones (two clones each from hygromycin B selectants and G-418 selectants) (supplementary material Fig. S8). After 2 hours, little accumulation of fluorophore above the kinetoplast region could be observed in these cells (Fig. 7A). Interestingly, about 37% (109 cells out of 294 cells that failed to traffic the endocytosed dye) of such Myo21<sup>+/–</sup> cells showed fluorophore accumulation in a crescent form at the flagellar-pocket region, which, to some extent, resembled the ‘big-eye’ phenotype, indicating defective endocytosis (Allen et al., 2003). This feature corresponded well with the excessive enlargement of some of the flagellar pockets (12%, 7 out of 57 enlarged flagellar pockets) observed during ultrastructural analyses (Fig. 6).

As an additional marker for intracellular trafficking, we also estimated secreted acid phosphatase (SAP) activity, which has been widely used for assessing the exocytic activity in these organisms (Overath et al., 1997; Cuvillier et al., 2000). SAP activity in the culture supernatants of the Myo21<sup>+/–</sup> mutant was significantly lower than that of wild-type cells (Fig. 5D, E). Further, we estimated a decrease in the relative distribution of SAP activity in Myo21<sup>+/–</sup> compared with wild-type cells (280-340 nm diameter, n=59) (Fig. 6), suggesting a defect in exocytic activity in these cells (Fig. 6).

**Fig. 5. Analysis of PFR in wild-type, Myo21<sup>+/+</sup> and Myo21<sup>+/–Comp</sup> cells.**

(A) Immunofluorescence images of wild-type (+/+), Myo21<sup>+/+</sup> (+/-) and Myo21<sup>+/–Comp</sup> (+/-) cells, labeled for paraflagellar rod (PFR, red), showing absence of PFR in Myo21<sup>+/+</sup> cells, which was regained in Myo21<sup>+/–Comp</sup> cells. Nuclei and kinetoplasts are labeled with DAPI (cyan). Scale bars: 5 μm. (B) Western blot analysis of PFR protein expression in Myo21<sup>+/+</sup> cells. Lane 1, wild-type cells; lane 2, Myo21<sup>+/–Comp</sup> cells. (C) Electron micrographs of thin sections of the cells showing presence, absence and regain of PFR proteins, respectively. a, Coomassie-blue-stained PVDF membrane (40 μg protein per lane); b, western blot using anti-Myo21 and anti-PFR antibodies; c, western blot using anti-Leishmania actin antibodies after stripping. Scale bars: 100 μm. A total of 55 cross-sections (CSs) and 14 longitudinal sections (LSs) of wild-type cells, 47 CSs and 6 LSs of Myo21<sup>+/+</sup> cells, and 13 CSs and 7 LSs of Myo21<sup>+/–Comp</sup> cells were analyzed.
reduced with time, compared with levels in wild-type cells (Fig. 7B). However, these defects in intracellular trafficking were reversed to normal after episomal complementation. Together, these results clearly reveal that Myo21 is essentially required for intracellular trafficking in \textit{Leishmania} cells.

To examine whether the reduced vesicular trafficking in Myo21\textsuperscript{+/–} cells was due to the flagellar pocket abnormalities or due to the direct involvement of Myo21 in the intracellular transport, we incubated the live Myo21-GFP expressing cells for 1 hour with FM4-64 and then analyzed the incubated cells for association of Myo21 with the internalized dye (Fig. 7C). Interestingly, many cells that trafficked FM4-64 beyond the flagellar pocket, showed association of Myo21-GFP with the dye, indicating a direct involvement of Myo21 in the intracellular vesicle transport in \textit{Leishmania} cells. However, our attempts to examine the association of actin with the vesicles containing dye, were not successful because of loss of FM4-64 in the cell-permeabilization step during the process of actin labeling with anti-actin antibodies (Sahasrabuddhe et al., 2004). Nevertheless, actin was also present in the regions where Myo21 associated with the dye (Fig. 1A; supplementary material Fig. S1).

### Intracellular trafficking is impaired by disrupting actin dynamics

Single and double Cof gene mutants (Cof\textsuperscript{+/–} and Cof\textsuperscript{−/−}) of \textit{Leishmania} have been described to display phenotypic properties that are similar to those observed here with Myo21\textsuperscript{+/−} mutants (Tammana et al., 2008). We therefore analyzed growth-dependent Myo21 expression (supplementary material Fig. S9A) and intracellular Myo21 localization (supplementary material Fig. S9B) in Myo21\textsuperscript{+/−} cells. These experiments revealed that similarly to the Myo21\textsuperscript{+/−} cells, Myo21 expression did not alter with the growth stage in these cells. The flagellar localization also remained unchanged despite the absence of actin in the flagellum. These results suggested that both Cof and Myo21 are required during assembly of the flagellum. Since depletion of Cof is known to severely impair actin dynamics (Tammana et al., 2008) and dynamic
actin is required in myosin-based intracellular trafficking (Zheng et al., 2009; Cramer, 2008; Semenova et al., 2008), we also analyzed vesicular transport in Cof−/− cells (supplementary material Fig. S9C). These mutants, similarly to Myo21+/− cells, exhibited considerably reduced vesicular transport, strongly suggesting an involvement of the actin-based intracellular-trafficking activity of Myo21 in assembly of the Leishmania flagellum. To further examine whether actomyosin activity was also required in the assembly of the IFT particles at the flagellar base, we immunolabeled both the Myo21+/− and Cof+/− cells for IFT172, using T. brucei IFT172 antibodies, which have been used as a marker of IFT in trypanosomes (Absalon et al., 2008). These antibodies intensely labeled the proximal flagellar region immediately below the region where Myo21 was concentrated in both the wild-type and mutant cells (Fig. 8), suggesting that Myo21 controls the IFT distribution in Leishmania cells. To test this possibility, we analyzed the IFT172 distribution in Myo21+/− cells that possessed relatively longer flagellum (supplementary material Fig. S10A). The IFT172 was distributed beyond the proximal flagellar location of Myo21 in these mutant cells, indicating that Myo21 does not control the IFT distribution in Leishmania flagellum. Furthermore, as the patterns of IFT172 staining in both the wild-type and mutant cells were similar (Fig. 8), we inferred that Myo21-mediated intracellular trafficking does not affect the assembly of the IFT particles at the flagellar base. This was confirmed when we observed similar expression levels of IFT172 in both wild-type and mutant cells in western blot analyses (supplementary material Fig. S10B).

**Discussion**

Most eukaryotes express several isoforms of myosin that perform diverse cellular functions. However, Leishmania cells express only a single myosin isoform, Myo21, which preferentially localizes at the proximal region of the flagellum (Katta et al., 2009). In the present study we show that Myo21 associates with actin and is indirectly involved in the flagellar length regulation. We further reveal that depletion of Myo21 results in impairment of flagellar assembly and intracellular trafficking. These abnormalities are, however, reversed by episomal complementation. In addition, we show that, similarly to Myo21-depleted cells, the flagellum assembly and intracellular trafficking are also impaired in the Cof-depleted cells. Together, these results indicate that the trafficking activity of Myo21 is essentially required in assembly of the Leishmania flagellum.

Eukaryotic cells are endowed with a well-coordinated intracellular trafficking system consisting of microfilament and microtubule tracks powered by myosin proteins, and kinesin and dynein motors, respectively (Ross et al., 2008). In the flagellar compartment, only kinesin and dynein microtubule-based motors have been implicated in cargo transport during flagellar assembly (Scholey, 2003; Cole and Snell, 2009). The flagellar cargoes associate with intraflagellar transport (IFT) complexes that are transported from the flagellar base to the flagellar tip (anterograde) and from the flagellar tip to the base (retrograde), respectively, by the kinesin and dynein motors (Rosenbaum and Witman, 2002). As the flagellar proteome consists of a plethora of proteins (more than 300) (Broadhead et al., 2006) and the number of cargoes associated with the IFT identified so far is limited, we propose that other mechanisms also operate in the transport system within the flagellum. An example in support of this proposal comes through an interesting study in which an IFT mutant of T. brucei showed movement of a subflagellar organelle, ‘the flagella connector’, beyond the axoneme microtubules, raising the possibility of a new form of intraflagellar transport besides the IFT (Davidge et al., 2006). Similarly, transport of PFR proteins in the flagellar compartment is also perplexing (Ralston and Hill, 2008), especially in induced IFT RNAi mutants of T. brucei (Absalon et al., 2008), conveying the gaps in our current knowledge of protein transport in the flagellar compartment. Bridging these observations is the example of the actin-based motor protein myosin VIIa, which

![Fig. 8. Localization of IFT172 and Myo21 in Leishmania promastigotes.](image-url)
localizes in the connecting cilium, and is responsible for transport of ‘opsin’ from the inner to the outer segment of photoreceptor (Liu et al., 1997; Liu et al., 1999). Since the presence of actin in the flagella has already been reported in several eukaryotic organisms, including Leishmania (Muto et al., 1994; Yanagisawa and Kamuya, 2001; Minoura, 2005; Sahasrabuddhe et al., 2004; Broadhead et al., 2006), the flagellar localization of Myo21 and its association with actin indicate the possible role of the actomyosin system in the intraflagellar transport of protein cargoes.

Protein transport in relation to flagella formation can be segregated into two major steps: (1) transport of protein complexes from the cell body to the flagellar compartment, and (2) transport of these complexes along the flagellar length within this compartment. In the Leishmania flagellum, Myo21 localizes predominantly and stably at the proximal region, whereas rest of the flagellar Myo21 content is detergent labile. This means that the fixed and free Myo21 can interact with different protein entities, which could impart different functions to these two Myo21 subpopulations. At the proximal region, Myo21 is fixed through its tail domain (Katta et al., 2009), implying that the motor domain (head domain) is freely projected in the flagellar matrix. This organization would form a ‘myosin-crew’ in such a manner that its motor activity can propel actin and/or actin-bound cargoes into or out of the flagellar compartment depending upon their orientation in the crew. Earlier studies have shown that inhibition of anterograde IFT results in abrupt seizure of flagella formation owing to the lack of building blocks, whereas inhibition of retrograde IFT results in short swollen flagella filled with amorphous material, which fails to recycle to the flagellar base (Pedersen and Rosenbaum, 2008). The Myo21+/– mutant, which possesses a short flagellum, shows no accumulation of material in the flagellar compartment and appears to be largely an anterograde-transport-inhibition phenotype. It would therefore seem that the reduction in myosin level might have impeded the entry of some of the cargos from the cell body to the flagellum.

To some extent the ‘myosin-crew’ model explains how the reduction of Myo21 would have affected the transport of many flagellar components from the cell body to the flagellum, and consequently, generation of the short-flagella phenotype. Disruption of a putative retrograde transport motor, DHC2.2, in L. mexicana is known to result in the short-flagella phenotype, but in contrast to other cell types (Pazour et al., 1999; Wicks et al., 2000), it does not lead to accumulation of flagellar proteins in its short flagellum (Adhiambo et al., 2005). We therefore speculate that in addition to anterograde transport, Myo21 might also be involved in the retrograde transport of the flagellar proteins within the flagellar compartment.

It has been suggested that IFT cargoes assemble at the base of the flagellum before being transported within the flagellar compartment (Cole and Snell, 2009; Bloodgood, 2000). Indeed, presence of IFT172 in the rudimentary flagellum in both Myo21+/– and Cof1+/– mutants suggests that assembly of IFT components is, in fact, not affected in these mutants. Nevertheless, it is possible that despite fully functional IFT-dependent transport, flagellum assembly is hampered because of the absence of components that are transported through the Myo21-dependent pathway. Alternatively, since flagellar elongation requires coordination of a number of interdependent signaling events (Rotureau et al., 2009), lack of key proteins transported through Myo21 might impede the cascade of flagella elongation and results in the short-flagella phenotypes, as observed in the present study. Furthermore, the detergent-soluble sub-fraction of Myo21 could be involved in the transport of proteins within the flagellum. It is, however, difficult to envisage at the moment whether this Myo21 subpopulation is part of the IFT or whether it forms a separate trafficking unit within the flagellum.

It has been reported that alterations in the lipid composition of the flagellar membrane hamper flagella formation (Tull et al., 2004). However, it is not yet clear whether axoneme assembly and the mobilization of lipid and protein components of the flagellar membrane are linked, or whether axoneme disassembly causes the concerted retrieval of the flagellar membrane (Cole and Snell, 2009). In the present study, Myo21 activity in membrane trafficking during endocytosis and exocytosis appear to link flagellar elongation with the addition of flagellar membrane and vice versa in trypanosomatids. The fact that Myo21 performs different functions at different locations raises the possibility of modulation in cargo recognition owing to post-translational modifications and/or other binding partners such as myosin heavy-chain kinases and calmodulin, both of which are encoded in the Leishmania genome. In addition, a distinct ubiquitin-associated domain at the C-terminus of Myo21 could also integrate extracellular signals and intracellular transport activities in these parasitic organisms.

Materials and Methods

Cell culture

L. donovani cells were maintained in high-glucose DMEM supplemented with 10% heat-inactivated fetal bovine serum and 40 μg/ml gentamycin at 25°C. To define the growth phases of Leishmania promastigotes, 105 cells/ml were seeded from the fully grown promastigote cultures and their growth was subsequently monitored by counting the cell numbers at 24 hour time intervals for at least 15 days. Cell growth was plotted on a log scale and early-log, mid-log and stationary phases were assigned in the curve (supplementary material Fig. S5). Sticking strictly to these growth conditions, the following parameters for early-log, mid-log and stationary phases of cell growth were used: 24-36 hours (0.5-1×106 cells/ml) as early-log phase, 60-96 hours (2-10×106 cells/ml) as mid-log phase and 12-14 days (~4×106 cells/ml) as stationary phase. Although mutant cells grew a little more slowly, they were also harvested at the same time points.

Deletion, constructs and genetic manipulation

The 5′ and 3′ flanking sequences Myo21 in L. donovani were determined using primer pairs F1, R1 and F2, R2 by PCR, as described earlier (Tammana et al., 2008). The amplicons were cloned in InstAclone vector (T257R, MBI Fermentas), and sequenced and submitted to EMBL (accession numbers, 5′ flank, FMS82168; 3′ flank, FMS82169). To generate deletion cassettes, the 5′ flank was amplified using primers F3 and R3 and cloned in T257R vector and named 5flk/T257R. Similarly, the 3′ flank was amplified using primers F4 and R4, cloned in T257R vector and named 3flk/T257R. Then the 3′ flank was digested at Nhel and BamHI sites and ligated in the 5′ flk/T257R after digestion with the same set of restriction enzymes. This clone containing both the 5′ and 3′ flanks was named 5/3flk/T257R. The 5′ end of the 3′ flank contained Nhel and NotI restriction sites, which were used for cloning of neomycin phosphotransferase (Neo) or hygromycin phosphotransferase (Hys) genes. Neo and Hys genes were amplified using F5, R5 and F6, R6 primer sets from pXG-GFP (a kind gift from Stephen M. Beverley) (Ha et al., 1996) and pCDNA3 vectors (Invitrogen) respectively, and cloned at Nhel and NotI site of 5/3flk/T257R. Both Hys and Neo genes were tagged with M-sequence (Debrabant et al., 1995) for improved expression of the Neo and Hys genes. Transfection of Leishmania promastigotes was carried out by electroporation of the deletion cassette released by Apel and Xhol digestion, as described earlier (Tammana et al., 2008).

The Myo21 single and double deletion mutants (Myo21+/– and Myo21−/–) of L. donovani were selected against 10 μg/ml G-418 and/or 10 μg/ml hygromycin on DMEM-agar plates. Single colonies on the plates were carefully picked and inoculated in liquid medium with appropriate antibiotics. Leishmania ADF/cofilin (Cof) single and double deletion mutants were generated as described earlier (Tammana et al., 2008). See supplementary material Table S1 for all primer sequences.

Scanning and transmission electron microscopy

For scanning electron microscopy, cells were fixed with 4% paraformaldehyde and 1% glutaraldehyde in 0.1 M phosphate-buffered saline (PBS), pH 7.4, and the cell suspensions were placed on poly-L-lysine (0.1%)-coated coverslips and allowed to adhere for 45 minutes in a humid chamber. Cells were post fixed in 1% osmium tetroxide for 1 hour at room temperature and were dehydrated in an ascending series of ethanol, critical-point dried and coated with Au-Pd (80:20) using a sputter coater.
Antibodies, western blotting and immunofluorescence

Ant Myo21 antibodies and anti-Leishmania actin antibodies were generated as described earlier (Katta et al., 2009; Sahasrabuddhe et al., 2004). Anti-PFR, anti-GRP78 and anti-IFT172 antibodies were a kind gift from Diane McMahon Pratt (Yale University, New Haven, CT), Emanuela Handman (Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia) and Philippe Bastin (Pasteur Institute-CNRS, Paris). anti-tubulin antibodies (1:1000) were from Sigma. For western blotting, lysates of promastigotes (Yale University, New Haven, CT), Emanuela Handman (Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia) and Philippe Bastin (Pasteur Institute-CNRS, Paris) were used. For western blotting, lysates of Leishmania promastigotes were prepared by washing the cells in PME buffer (0.1 M PIPES, 1 mM MgCl2 and 1 mM EGTA) and boiling the washed cells in 1× SDS sample buffer. Protein samples (cells lysates, 40 μg/lane) were resolved on SDS-PAGE and electroblotted on to a PVDF membrane. The blotted membranes were treated with blocking buffer (5% skimmed milk in PBS) and probed with the primary antibodies [actin; 1:10,000; Myo21; 1:1000; mAb10E2 (Ismach et al., 1989), 1:1000; GRP78 (Jensen et al., 2001), 1:100,000 and IFT172 (Absalon et al., 2008), 1:2000] diluted in blocking buffer. HRP-conjugated secondary antibodies (Santa Cruz) were used at 1:20,000 dilution. The protein bands were detected using Millipore chemiluminescent reagent and band intensities were measured using image master software.

For immunofluorescence microscopy, cells were washed with PBS and attached to glass coverslips coated with poly-L-lysine for 5 minutes at 25°C and then fixed with 4% (v/v) paraformaldehyde in PBS for 15 minutes. Fixed cells were permeabilized with 0.5% NP-40 and blocked with 1% BSA in PBS. For the preparation of the cytoskeleton, cells attached to the poly-L-lysine-coated coverslips were fixed with 4% (w/v) paraformaldehyde in PBS for 15 minutes. Fixed cells were washed with chilled PME buffer and then fixed with 4% (w/v) paraformaldehyde in PBS as above. Cells were treated with anti-Myo21 antibodies (1:1000), anti-actin antibodies (1:500) and/or anti-tubulin antibodies (1:1000) for 4 hours at 4°C and stained with Alexa Fluor 488- and Cy3-labelled secondary antibodies (1:1000). To label IFT172, cells on poly-L-lysine-coated coverslips were fixed for 5 minutes in methanol at −20°C, blocked in 0.1% BSA in PBS for 10 minutes (Absalon et al., 2008) and processed for labelling, as above. Image acquisition was done on ZEISS LSM510 META confocal system using a 63×/1.4 NA (OIL) Plan Apochromate lens and 3× digital zoom. Since the fluorescence signal of Myo21 was very low in other regions of the cell body compared with the proximal region of the flagellum, immunofluorescence imaging was performed at relatively high gain settings in cases when Myo21 needed to be visualized clearly at the proximal region of the flagellum, immunofluorescence imaging was performed at relatively high gain settings in cases when Myo21 needed to be visualized clearly at the proximal region of the flagellum, immunofluorescence imaging was performed at relatively high gain settings in cases when Myo21 needed to be visualized clearly at the proximal region of the flagellum, immunofluorescence imaging was performed at relatively high gain settings in cases when Myo21 needed to be visualized clearly at the proximal region of the flagellum, immunofluorescence imaging was performed at relatively high gain settings in cases when Myo21 needed to be visualized clearly at the proximal region of the flagellum, immunofluorescence imaging was performed at relatively high gain settings in cases when Myo21 needed to be visualized clearly at the proximal region of the flagellum, immunofluorescence imaging was performed at relatively high gain settings in cases when Myo21 needed to be visualized clearly at the proximal region of the flagellum, immunofluorescence imaging was performed at relatively high gain settings in cases when Myo21 needed to be visualized clearly at the proximal region of the flagellum, immunofluorescence imaging was performed at relatively high gain settings in cases when Myo21 needed to be visualized clearly at the proximal region of the flagellum, immunofluorescence imaging was performed at relatively high gain settings in cases when Myo21 needed to be visualized clearly at the proximal region of the flagellum, immunofluorescence imaging was performed at relatively high gain settings in cases when Myo21 needed to be visualized clearly at the proximal region of the flagellum, immunofluorescence imaging was performed at relatively high gain settings in cases when Myo21 needed to be visualized clearly at the proximal region of the flagellum, immunofluorescence imaging was performed at relatively high gain settings in cases when Myo21 needed to be visualized clearly at the proximal region of the flagellum.

Motility assessment by time-lapse microscopy

Motility measurements were performed on a Leica microscope DM5000B after collecting time-lapse movies for 36 seconds at 10× magnification. Paths of individual cells displayed on the time-lapse movie were traced manually on a transparency and scanned for presentation (Leica, Germany) as described earlier (Tammanna et al., 2004). Briefly, videos were converted into 8-bit grey-scale movies and then processed for labelling, as above. Image acquisition was done on ZEISS LSM510 META confocal system using a 63×/1.4 NA (OIL) Plan Apochromate lens and 3× digital zoom.

Intracellular trafficking

The intracellular trafficking in Leishmania, endocytic vesicles were traced microscopically by using the fluorophore FM4-64, essentially as described (Sahin et al., 2008; Mullins et al., 2001). Briefly, Leishmania cells (5×106 cells/ml) were treated with FM4-64 dye in a microcentrifuge tube at 4°C for 10 minutes and the temperature was then raised to 25°C. Small aliquots (50 μl) were then harvested at different time intervals and mixed with 2% paraformaldehyde solution prepared in phosphate-buffered saline. To the fixed, labeled cells Hoechst 33342 dye was added at 10 μg/ml and the cells were immediately imaged under Zeiss LSM510 Meta confocal microscope using a 63×, 1.4 NA (OIL) Plan Apochromate lens and 3× digital zoom.

The exocytic activity was assessed by measuring SAP in the Leishmania culture medium at different time intervals, as described (Bakalar et al., 1995). Briefly, cells at 10 cells/ml density were resuspended in a 15 ml culture medium and 1 ml of the culture was harvested at 24 hour time intervals for 7 days. Cells were centrifuged and the pellets stained with formaldehyde and acetic acid. The pellets were analyzed under a FEI Tecnai-12 Twin Transmission Electron Microscope equipped with a SIS Mega View II CCD camera at 80 kV (FEI Company).

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Supplementary Fig. S1

(A) Immunofluorescence images of whole cells (WC) and cytoskeletons (CSK) of *Leishmania* promastigotes labeled with anti-Myo21 antibodies (green) and anti-*Leishmania* actin antibodies (red). Images captured at the same gain/offset settings to show the presence of Myo21 throughout the flagellum of whole cells, and its restricted presence only in the proximal region of the flagellum in the cytoskeleton. Bar, 5µm. Arrowheads mark co-localization of Myo21 and actin at different locations in the cell body.

(B) Immunofluorescence image of a *Leishmania* promastigote labeled with anti-Myo21 (green) and anti-actin (red) antibodies with increased brightness and contrast showing co-localization of Myo21 with actin in the cell body.

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(B) Immunofluorescence image of a *Leishmania* promastigote labeled with anti-Myo21 (green) and anti-actin (red) antibodies with increased brightness and contrast showing co-localization of Myo21 with actin in the cell body.
Supplementary Fig. S10

(A) Immunofluorescence microscopic analysis of IFT172 localization in Myo21\(^{+/−}\) cells containing relatively longer flagella. Distribution of IFT172 beyond the proximal flagellar region Myo21\(^{+/−}\) cells shows no alteration in IFT172 distribution in the mutant cells, compared to the wild type parasite. IFT172, red; Myo21, green. Bar, 5 µm.

(B) Western blot analyses of wild type (lane 1) and Myo21\(^{+/−}\) (lane 2) cells using anti-IFT172 antibodies, showing similar amounts of IFT172 in both the cell types (50 µg protein per lane). The blot was stripped and sequentially reprobed with anti-Myo21 and anti-actin antibodies to assess the Myo21 expression in the mutant, using actin as an internal loading control. Note, both monoclonal and polyclonal antibodies against *Trypanosoma brucei* IFT172 faintly cross-reacted with some *Leishmania* proteins other than the IFT172-like protein (199 kDa) in *Leishmania* promastigote lysate.

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Fig. S2
Semi-quantitative Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) analyses of the mRNA extracted from early-log phase and stationary phase cells for expression of Myo21. (A) The PCR reactions were harvested at different number of cycles as indicated and electrophoresed on 1% agarose gel. (B) Spectroscopic analyses (OD\textsubscript{260}) of the increase in the amplified DNA, eluted from the DNA bands in ‘A’. Values are plotted after normalization with their respective actin bands as internal control.
Fig. S3
Southern blot analysis of the genomic DNA of wild type *Leishmania donovani* cells after digestion with Apal, XhoI and Sacl. *Myo21* gene contains one restriction site for Sacl at 2312 bp, which typically gives two bands on the blot unlike, Apal and XhoI, suggesting single copy architecture of *Myo21* gene in the *L. donovani* genome.
Fig. S4
Analyses of ploidy generation in Myo21\textsuperscript{-/-} clones. A, Southern blot analyses of a Myo21\textsuperscript{+/-} clone and five Myo21\textsuperscript{-/-} clones showing replacement of the Myo21 gene with Hyg and Neo genes and also the presence of Myo21 gene containing chromosome in the Myo21\textsuperscript{-/-} clones; B, western blot analyses of the expression of Myo21 in five Myo21\textsuperscript{-/-} clones; C, Flow-cytometry analysis of wild type, Myo21\textsuperscript{+/-} (Hyg), Myo21\textsuperscript{+/-} (Neo) and Myo21\textsuperscript{-/-} cells showing similar DNA contents. +/+, wild type cells; +/-Neo, neomycin resistant heterozygous Myo21 mutant; +/-Hyg, hygromycin resistant heterozygous Myo21 mutant; -/-, homozygous Myo21 mutant.
Supplementary Fig. S5

Growth curve of wild type cells plotted in logarithmic scale. Time-scale at which the cells were harvested for experiments, are indicated.

- 24-36 hour (0.5-1 x 10^6 cells/ml) as early-log phase,
- 60-96 hour (2-10 x 10^6 cells/ml) as mid-log phase and
- 12-14 days (~4 x 10^7 cells/ml) as stationary phase.

Fig. S5
Growth curve of wild type cells plotted in logarithmic scale. Time-scale at which the cells were harvested for experiments, are indicated.
Fig. S6
Histograms of flagellar length of two independently isolated neomycin resistant heterozygous clones (+/-Neo1 and +/-Neo2) and an additional hygromycin resistant clone (+/-Hyg2) of Myo21 mutants. More than 100 cells were analysed from each cell line.
Supplementary Fig. S7

Intracellular distribution of Myo21 in Myo21^{+/-} cells.

(A) Immunofluorescence images of wild type (+/+) and Myo21^{+/-} (+/-) cells labeled with anti-Myo21 and anti-Leishmania actin antibodies, showing dot-like appearance of Myo21 at the proximal region of the residual flagellum. Bar, 5µm.

(B) Western blot analyses of the detergent soluble and insoluble fractions of wild type and Myo21^{+/-} cells showing similar distribution of Myo21 in both the cell types. Lanes 1 and 3, NP-40 insoluble fractions; lanes 2 and 4, detergent soluble fractions of wild type and Myo21^{+/-} cells respectively. Wild type and Myo21^{+/-} cells (5 x 10^7) cells were extracted in presence of 1.0% (v/v) NP-40 in a total volume of 100 µl. 10 µl each of soluble (~30 µg protein) and insoluble (~20 µg protein) fractions were subjected to western blot analyses. The blots probed with anti-Myo21 antibodies were stripped and reprobed with anti-PFR antibodies (marker for cytoskeleton) and anti-GRP78 antibodies (marker for cytosol) to show the purity of the preparations.
Fig. S8
Quantitative analyses of wild type as well as two each of randomly selected hygromycin (Hyg1 and Hyg2) and neomycin resistant (Neo1 and Neo2) Myo21^+/- cells, showing percent of total cells internalizing of FM4-64 dye beyond the nucleus in 1 hour, as judged by fluorescence microscopy. At least 600 cells each of the mutants were subjected to the analysis.
Fig. S9
Expression, intracellular distribution and intracellular trafficking analyses of Myo21 in LdCof⁻/⁻ (LdCof null) cells.
(A) Western blot of LdCof⁻/⁻ cells at different growth phases showing no variation in expression of Myo21 in these cells (40 µg protein per lane). e-log, early-log phase cells; m-log, mid-log phase cells; stat, stationary phase cells.
(B) Immunofluorescence images showing localization of Myo21 in the residual flagellum in LdCof⁻/⁻ cells. Absence of actin in the residual flagellum is apparent and intracellular actin cables are clearly visible in LdCof⁻/⁻ cells. Fluorescence signals are not quantitative. Bar, 5µm.
(C) Microscopic analyses of uptake of FM4-64 dye showing significantly reduced intracellular vesicular trafficking in LdCof⁻/⁻ cells as compared to wild type cells. Kinetoplast (K) and nucleus (N) are stained with Hoechst dye as intracellular markers. Bar, 5µm.
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