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Report

A Novel Microtubule-Depolymerizing Kinesin Involved in Length Control of a Eukaryotic Flagellum

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Summary

Cilia and flagella are complex, microtubule (MT)-filled cell organelles of which the structure is evolutionarily conserved from protistan cells to mammalian sperm and the size is regulated [1]. The best-established model for flagellar length (FL) control is set by the balance of continuous MT assembly and disassembly occurring at the flagellar tip [2, 3]. Because steady-state assembly of tubulin onto the distal end of the flagellum requires intraflagellar transport (IFT)—a bidirectional movement of large protein complexes that occurs within the flagellum—FL control must rely upon the regulation of IFT [4, 5]. This does not preclude that other pathways might "directly" affect MT assembly and disassembly [4]. Now, among the superfamily of kinesins, family-13 (MCAK/KIF2) members exhibit a MT-depolymerizing activity responsible for their essential functions in mitosis [6]. Here we present a novel family-13 kinesin from the flagellated protozoan parasite *Leishmania major*, that localizes essentially to the flagellum, and whose overexpression produces flagellar shortening and knockdown yields long flagella. Using negative mutants, we demonstrate that this phenotype is linked with the MT-binding and -depolymerizing activity of this kinesin. This is the first report of an effector protein involved in FL control through a *direct* action in MT dynamics, thus this finding complements the assembly–disassembly model.

Results and Discussion

The *LmjKin13-2* Gene Encodes an "Ancestral" Family-13 Kinesin

Leishmania and *Trypanosoma* are uniflagellated protozoa belonging to the family Trypanosomatida and are responsible for a wide spectrum of human and animal diseases. They have recently appeared as suitable model organisms for the study of eukaryotic flagella [7, 8]. The recent completion of their genome-sequencing programs [9, 10] also revealed that they exhibit an unusually high complement of kinesins, with 54 putative kinesins, of which five are undoubtedly related to the microtubule (MT)-depolymerizing kinesin-13 family ([11] and unpublished data). The first protein of this kinesin-13 family on which we focused was a mitotic-centromere-associated kinesin (MCAK)-like protein that, as expected, localized to the nucleus and was involved in mitosis [12]. The second one, also annotated as MCAK-like in the genome database GeneDB (http://www.genedb.org) and here termed LmjKIN13-2, is encoded by gene *LmjF13.0130* (EMBL accession number CT005252.1). It is a 730 amino acid protein that contains the highly conserved kinesin motor domain in internal position (residues 40–356 according to Pfam, score 8.2e-112). The alignment of the motor-domain sequence with that of nine other kinesin-13 members revealed the conservation of residues and motifs previously identified as strictly specific of the kinesin-13 family and involved in their depolymerizing activity (Figure S1 in the Supplemental Data available online) [13, 14], in particular the KVD site (here KLD) necessary for MT depolymerization and the KEC site apparently essential for binding to MTs. This clearly classifies this protein among kinesin-13 members and makes it different from previously identified flagellar kinesins—kinesin-II, the ubiquitous molecular-motor-driving anterograde intraflagellar transport (IFT) [5], and KLP1, essential for flagellar motility [15]—that both belong to other kinesin families and are also present in trypanosomatids. Surprisingly, however, the sequence of the "neck" domain of the protein, adjacent to the motor domain and considered to be conserved in kinesin-13s [16], is not conserved here. Sequence alignments show that this supposedly family-specific neck sequence is not present in the two other kinesin-13s from protozoa that have been published [12, 14], in the kinesin-13 from *Chlamydomonas*, or in the other kinesin-13s of *Leishmania* (Figure S1, unpublished data). A recent phylogenetic analysis of the kinesin superfamily could distinguish two groups in kinesin-13s: the "animal-specific" MCAK/KIF2 subfamily and the ubiquitous and more "ancestral" KIF24 subfamily [17]. All protistan members of the kinesin-13 family included in this phylogenetic study belong to the latter. Our own alignment of KIF24 with MCAK subfamily members again failed to identify a conserved neck sequence in KIF24 (not shown), suggesting that this feature is actually not part of the KIF24 subfamily.

The next most closely related family to kinesin-13s is the kinesin-8 family, which exhibits both a plus-end-directed MT-depolymerase activity and a translocation activity but does not possess the kinesin-13-specific motifs. Interestingly, whereas the less "ancestral" yeast *Saccharomyces cerevisiae* lacks kinesin-13 family members and hence appears to only rely upon kinesin-8s for MT depolymerization [18], *L. major* lacks kinesin-8 members [11] but has more kinesin-13s—suggesting

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that different organisms have evolved varying strategies for performing similar cell-biological functions relying upon kinesins.

LmjKIN13-2 Localizes to the Flagellum

The *LmjKin13-2* gene was introduced into the expression vectors pTH6nGFPc and pTH6cGFPn [12]. After transfection into *L. major* cells, both vectors are maintained episomally and allow the constitutive expression of a recombinant protein bearing the GFP either at the N- or the C-terminal end. Surprisingly, expression of both GFP-fused proteins allowed their visualization essentially at the distal tip and the basis of the flagellum and, when more pronounced, along the length of the flagellum, the cytoplasm being only slightly decorated (Figure 1, Movie S1). When we substituted the GFP with the less bulky c-Myc tag, the localization, revealed by immunofluorescence, proved similar, whether the tag was in the N- or C-terminal position (Figure S2). The recombinant protein was never observed at the nucleus level, particularly at the mitotic spindle (Figure S3), despite its primary annotation as an MCAK. Moreover, no phenotypic changes concerning mitosis or in vitro cellular growth were observed, whether in *L. major* cells overexpressing the protein or in *T. brucei* cells subjected to RNAi (see below), strongly suggesting that LmjKIN13-2 is not involved in mitotic division.

LmjKIN13-2 Overexpression Induces Short-13 Flagellum Phenotypes

The second remarkable observation with this recombinant expression was that more than 90% of the cells exhibited a phenotype consisting of a significant reduction of the flagellar length (FL) (Figures 1, 2A, and 2B). Thus, the FL in the mid-log growth phase was reduced to 52% and 70% of its value in the wild-type strain in the cell lines expressing LmjKIN13-2-GFPc and GFPn-LmjKIN13-2, respectively ($p < 0.0001$ in both cases) (Table S1). This was completed by a modification of the cell morphology, with a short, and often stumpy, cell body (Figure 1). Identical phenotypes were obtained with the c-Myc-tagged protein and, more importantly, with the nontagged full-length recombinant protein (Table S1). This shows that the phenotype was not caused by protein-function impairment due to end tagging. Also of note is the fact that flagellum motility was retained in the mutant cells (Movie S1). All these data strongly suggest that LmjKIN13-2 is a flagellar protein involved in FL control.

Flagella are dynamic organelles that undergo continuous turnover [2]. FL is thought to be primarily controlled through changes in the ratio of IFT-dependent tubulin assembly versus disassembly and hence through the regulation of IFT [1, 2, 4]. This model was supported by the short-flagellum phenotypes obtained a variety of systems including trypanosomes, after mutations affecting IFT proteins (reviewed in [19, 20]). Nevertheless, this does not preclude a complementary regulation model, also partly supported by the analysis of short-flagellum mutants [7, 21–27] and based on a signaling pathway yet to be identified (see below). Here, we report the effect of a protein that, being a kinesin-13, can directly promote flagellar disassembly by catalyzing MT depolymerization [6].

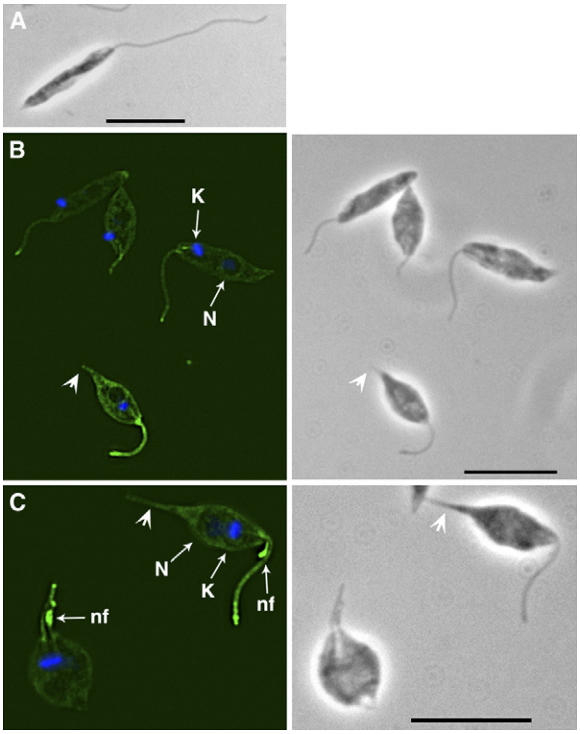

Figure 1. LmjKIN13-2 Shows an Unusual Localization at the Flagellum and Induces Short-Flagellum Phenotypes

(A) Typical morphology of a wild-type *L. major* "Fiedlin" promastigote cell in log-phase growth viewed in phase-contrast microscopy. The scale bar represents 10 μm.

(B) Images of *L. major* cells expressing LmjKIN13-2-GFPc (the protein fused to GFP at its C-terminal end) viewed in fluorescence (left) and phase-contrast (right) microscopy. LmjKIN13-2-GFPc (green) localizes essentially at the flagellum. The nucleus (N) and the kinetoplast (K), which is the single mitochondrial DNA located near the basal body of the flagellum, are stained with DAPI (blue). The mutant cells exhibit demonstrative morphological phenotypes, with a shortened flagellum and a short and often ovoid cell body, as well as, in some cells, an elongation of the posterior end (thick arrowhead). The scale bar represents 10 μm.

(C) Images of *L. major* cells expressing GFPn-LmjKIN13-2. Phenotypes in this line were similar to those observed in (B). The cells exhibiting the most intense flagellar fluorescence were mainly predividing and dividing cells (visible here from the presence of the nascent daughter flagellum, nf). N indicates the nucleus and K indicates the kinetoplast. The scale bar represents 10 μm.

Flagellum Shortening Is Due to the Depolymerizing Activity of LmjKIN13-2

All members of the kinesin-13 family that could be tested to date, and particularly their representative members, XKCM1 in *Xenopus* [28, 29] and MCAK in mammals [30, 31], have been shown to effectively depolymerize MTs in vitro. The same has been found for the protozoan PfKinI of *Plasmodium falciparum* [14] as well as for the mammalian KIF2A, which apparently plays a nonmitotic role in the development of the nervous system by suppressing extension of superfluous branches at the cell edge of postmitotic neurons [32]. In the kinesin-13 family, at least two class-specific motifs have been described within the motor domain as essential for MT-depolymerizing activity. The most significant one is the KVD finger. Mutational studies on the PfKinI catalytical core, exchanging KVD for three alanine residues,

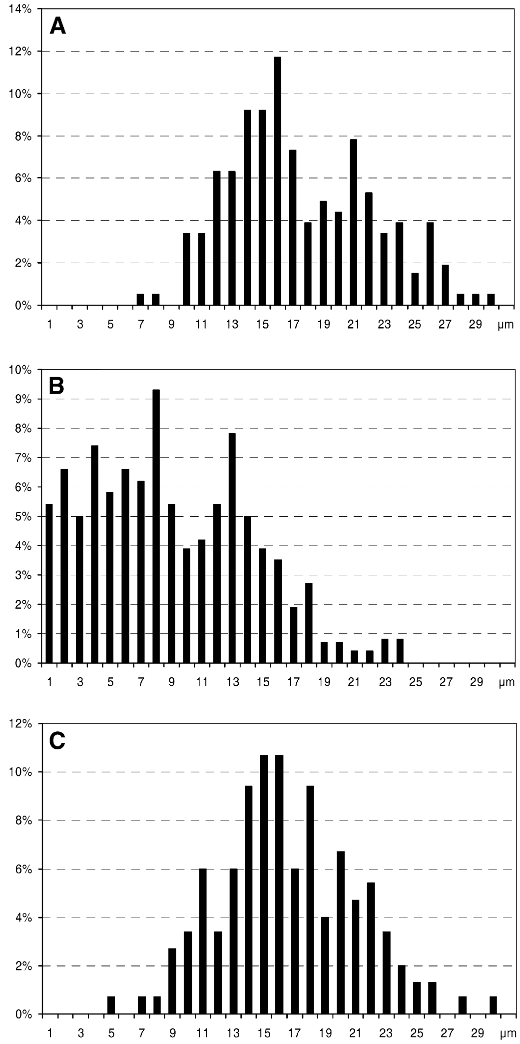

Figure 2. Histograms of Flagellar Lengths in Wild-Type and Mutant *L. major* Promastigote Cells

(A) Wild-type Friedlin cell line. The abscissa shows groups of flagellar lengths in microns and the ordinate shows the number of cells as percentages. Typically, 200 flagella were measured for each cell line. All flagella were measured at the same stage of growth (see Supplemental Data).

(B) Transformant cloned cell line expressing LmjKIN13-2-GFPc, showing a very high proportion of short flagella.

(C) Transformant cloned cell line expressing LmjKIN13-2-GFPc mutated at the KLD site (ΔKLD/AAA), showing restoration of the wild-type phenotype.

completely abolished depolymerizing activity, whereas the ATPase and MT-binding activities were intact. A second class-specific set of residues, the KEC motif, was also shown to be essential for depolymerization through MT binding [14].

We therefore mutagenized these two sites in LmjKIN13-2 and expressed the mutant proteins as GFP-fusion proteins in *L. major.* The replacement of the first motif (here KLD, position 75 of the motor domain [MD]) by three alanines was sufficient to restore wild-type morphology (Figures 2C and 3A, Table S1). This mutation also caused the loss of localization of the mutated protein in the flagellum, this protein being located only at the flagellar base. Alanine replacement in the KEC motif (position 292 of the MD) partially restored the wild-type phenotype and gave the same localization as the KLD mutation (Figure 3B, Table S1). With each of these mutations, the flagellum was significantly longer than the short-flagellum phenotype caused by the expression of the full-length recombinant kinesin ($p < 0.0001$). These results show that MT depolymerization is the basis for the observed effect of LmjKIN13-2 on FL.

Because the neck of kinesin-13s is known to be essential for efficient MT depolymerization in mammalian MCAKs [13, 16], we also constructed a mutant where most of the N-terminal part of LmjKIN13-2 (residues 1–30) was deleted. A GFP-fused version of this mutant localized to the flagellum like the full-length recombinant LmjKIN13-2 (not shown), and its overexpression also yielded a short-flagellum phenotype (Table S1). This suggests that, as suspected for *P. falciparum* PfKinI [33], this domain may not be essential for the depolymerizing activity of this kinesin—a hypothesis that might apply to other "ancestral" kinesin-13s of the KIF24 subfamily.

RNAi Knockdown of the Ortholog of *LmjKIN13-2* Induces an Increase in Flagellar Length

RNA interference (RNAi) is not functional in *Leishmania* but is efficient in *T. brucei*. Therefore, we constructed an RNAi vector to inhibit the expression of *Tb11.02.2260*, the exact ortholog of *LmjKIN13-2* in *T. brucei* (see Supplemental Data). From 4 days of induction, a significant increase of the mean FL was observed as compared with noninduced transformants (Figure 4). No in vitro cell-growth impairment was noted (not shown). In order to control for possible off-target effects of the RNAi, we performed a second RNAi experiment directed to another portion of the gene that does not overlap with the portion used in the first experiment. A similar FL increase was then obtained (Figure S4). These data strongly suggest that LmjKIN13-2 activity is present at steady state and therefore coexists with constitutive disassembly as well as IFT in a complex FL-control process. This also supposes some degree of regulation of this activity in order to maintain a balance between shortening and lengthening of the flagellum.

Long-flagellum phenotypes have been reported previously, essentially following mutations of protein-kinase genes [7, 21, 24, 26, 27]. Thus, the alternative model of FL control exposed above has gained support from the identification of a MAP-kinase cascade where loss of function yielded flagella of altered length, particularly in *Leishmania* [7, 24, 25, 27]. Similar data have been obtained in *Chlamydomonas* with a NIMA-related kinase that was found to regulate FL by promoting flagellar disassembly [34]. Nevertheless, the substrates of these kinases remain unknown. Kinesins are well known to be subject to regulation via specific phosphorylation events [6]. Hence, although this remains speculative, a future working hypothesis would be that LmjKIN13-2 be a substrate of such a regulating cascade.

The data presented here are novel in two ways. They are the first report of an MCAK-like protein localized to a eukaryotic flagellum. More importantly, they also constitute the first report of an effector protein that would be directly involved in the process of FL control, through

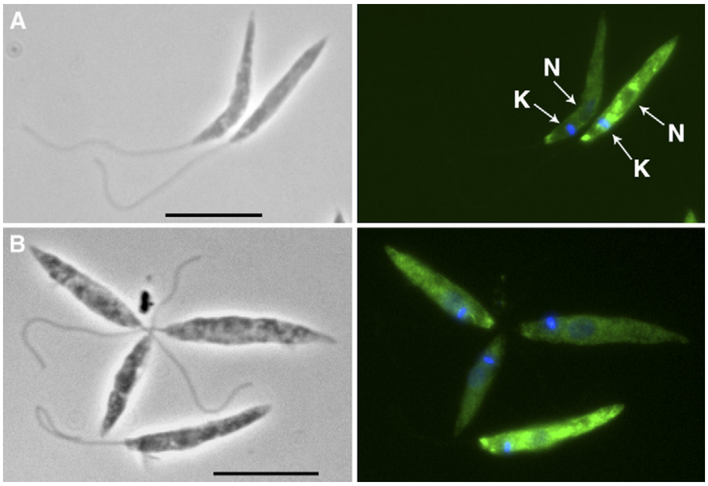

Figure 3. Mutations of LmjKIN13-2 at the KLD and KEC Sites Restore the Wild-Type Phenotype

(A) Images of *L. major* cells expressing LmjKIN13-2-GFPc mutated at the KLD site (ΔKLD/AAA) viewed in phase-contrast (left) and fluorescence (right) microscopy. Promastigotes exhibit typical wild-type long flagella and slender cell bodies. LmjKIN13-2-GFPc (green) did not decorate the flagellum but instead showed a cytoplasmic localization with preferential accumulation at the anterior pole of the cell. K indicates the DAPI-stained (blue) kinetoplast DNA and N indicates DAPI-stained nucleus. The scale bar represents 10 μm.

(B) Images of *L. major* cells expressing LmjKIN13-2-GFPc mutated at the KEC site (ΔKEC/AAA). The wild-type morphology appeared to be restored, and the phenotype is as in (A). A loss of localization at the flagellum was also noted. The scale bar represents 10 μm.

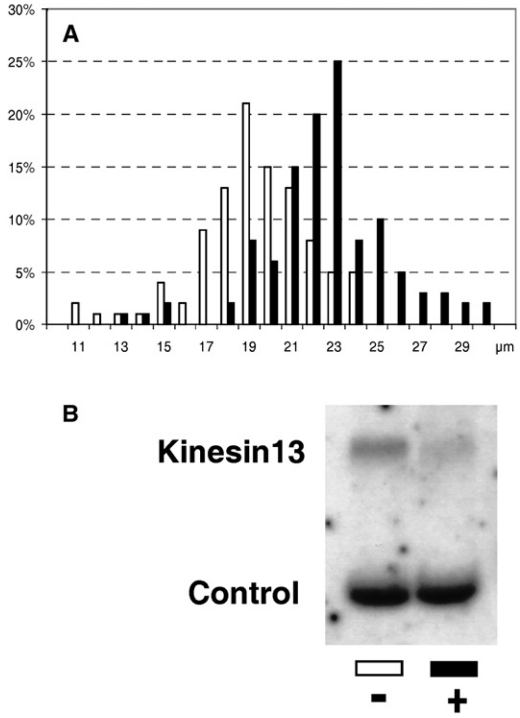

Figure 4. RNAi Knockdown of the Ortholog of *LmjKIN13-2* in *Trypanosoma brucei* Induces Flagellar Lengthening

(A) Histogram of flagellar lengths in tetracyclin-induced (black bars) and noninduced (white bars) *T. brucei* transfected procyclic cells. Abscissa and ordinate are as in Figure 2. The flagellar measurements shown here were made after 6 days of induction. Two hundred cells were examined for each experimental condition. The mean flagellar lengths for tetracyclin-induced versus noninduced transformants were 22.5 ± 2.9 μm and 19.3 ± 2.7 μm ($p < 0.0001$).

(B) Northern-blot analysis of RNAi of *Tb11.02.2260*, the ortholog of *LmjKIN13-2*, in *T. brucei* procyclic cells. Total RNA (10 μg/lane) isolated from transfected procyclic cells grown in the absence (−) or presence (+) of tetracyclin (1 μg/ml) for 5 days was electrophoresed, blotted, and probed at high stringency with a 454 bp and a 653 bp DNA fragment of the *Tb11.02.2260* gene (kinesin-13, top) and the *T. brucei* GPI8 anchor-subunit gene *Tb10.61.3060* (control, bottom), respectively.

the depolymerization of axoneme MTs. Obviously, more data need to be gathered before a complete picture of the part played by this protein in FL regulation emerges—e.g., its interactions with other known flagellar proteins or whether it is truly involved in a feedback system. Still, kinesin-13 family members are ubiquitous proteins with a conserved MT-depolymerizing function in almost all eukaryotes studied to date. Kinesin LmjKIN13-2 might thus be one of the missing pieces in the FL-regulation puzzle. Indeed, our data do not contradict but complement the existing FL-control models [1, 4] in an aspect based upon a direct intervention in MT dynamics.

Supplemental Data

Experimental Procedures, four figures, one table, and one movie are available at http://www.current-biology.com/cgi/content/full/17/9/778/DC1/.

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