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A non-mitotic CENP-E homolog in *Dictyostelium discoideum* with slow motor activity

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ABSTRACT

Kinesins are ATP-dependent molecular motors that mediate unidirectional intracellular transport along microtubules. *Dictyostelium discoideum* has 13 different kinesin isoforms including two members of the kinesin-7 family, Kif4 and Kif11. While Kif4 is structurally and functionally related to centromere-associated CENP-E proteins involved in the transport of chromosomes to the poles during mitosis, the function of the unusually short CENP-E variant Kif11 is unclear. Here we show that orthologs of short CENP-E variants are present in plants and fungi, and analyze functional properties of the *Dictyostelium* CENP-E version, Kif11. Gene knockout mutants reveal that Kif11 is not required for mitosis or development. Imaging of GFP-labeled Kif11 expressing *Dictyostelium* cells indicates that Kif11 is a plus-end directed motor that accumulates at microtubule plus ends. By multiple motor gliding assays, we show that Kif11 moves with an average velocity of 38 nm/s, thus defining Kif11 as a very slow motor. The activity of the Kif11 motor appears to be modulated via interactions with the non-catalytic tail region. Our work highlights a subclass of kinesin-7-like motors that function outside of a role in mitosis.

1. Introduction

Kinesins are molecular motors that mediate unidirectional intracellular transport along microtubule tracks in eukaryotes. The superfamily of kinesin motor proteins is subdivided into 14 subfamilies [1]. Discovered more than two decades ago as novel ATPases involved in microtubule based motility, kinesin subfamilies can be classified on the basis of their directionality (plus or minus end-directed movement on microtubules), by the localization of the catalytic domain (N-terminal, internal, or C-terminal), and by their variable polypeptide chain composition (monomers, homodimers, heterodimers) [2]. Functionally, kinesins are involved in different processes that include the transport of organelles, patterning of mRNAs during development, assembly of higher ordered structures such as mitotic spindles, and in the mechanics of chromosome movement during cell division.

Dictyostelium discoideum is a model eukaryote that has been used to study multiple aspects of development, differentiation, chemotaxis, and motility [3]. 13 kinesin isoforms have been identified in *Dictyostelium* [4]. 12 of these isoforms in *Dictyostelium* are grouped into nine defined kinesin subfamilies (kinesin-1 (Kif3, Kif5, Kif 7), -3 (Kif1), -4 (Kif8), -5 (Kif13), -6 (Kif12), -7 (Kif4, Kif11), -8 (Kif10), -13 (Kif6), and -14 (Kif2)) in addition to one architecturally distinct motor (Kif9) [5]. Functions of all 13 *Dictyostelium* kinesins have been analyzed by gene disruption methods (summarized in [5]). Interestingly, only Kif3 (a kinesin-1 organelle transporter homolog) and Kif6 (the kinesin-13 homolog) appear to be essential for viability. Disruption of *kif1* and *kif12* produce phenotypes suggesting functions similar to family isoforms in other organisms. Gene disruptions also revealed unexpected phenotypes that illustrate the evolutionary adaptability of the kinesin gene family. For example, the middle-motor kinesin Kif9 serves a critical role in connecting the centrosome to the nucleus during interphase [5,6], while the highly conserved kinesin-5 homolog is dispensable for cell division [7].

In contrast to most animal cells, *Dictyostelium* contains two distinct isoforms of the kinesin-7 family, Kif4 and Kif11. Both kinesins contain a highly conserved CENP-E motor domain followed by a downstream neck region, but differ strikingly in the length and arrangement of sequence in other regions. Kif4 is structurally homologous to the canonical long, CENP-E isoform associated with kinetochores, and serves as a plus end-directed motor for metaphase chromosome alignment [8]. Although the disruption of Kif4 in *Dictyostelium* is viable, null-mutants grow slowly, and when challenged with defects in dynein, display chromosomal aberrations consistent with a function similar to other CENP-E isoforms [9].

Further studies using single molecule measurements indicate that CENP-E is a slow, processive transport motor [10,11], and

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promotes microtubule plus-end elongation [12]. In contrast to CENP-E/Kif4, the other kinesin isoform Kif11 is significantly smaller in size and contains a short sequence region upstream of the conserved motor domain. Gene knockouts of Kif11 also indicate a function distinct from Kif4, whereby null-cells grow similar to wild type, either on bacteria or in shaking culture, and cell division is unaffected. In order to examine the functions of Kif11 in greater detail, we analyzed its localization *in vivo* and investigated the catalytic properties of the Kif11 motor. Similar shorter versions of kinesin-7 family members are also prevalent in plants, amoebae, and fungi [13]. These works and our own analyses here suggest a functionally distinct subdivision within the kinesin-7 family.

2. Materials and methods

2.1. Vector construction, D. discoideum cell culture and transformation

For the GFP-Kif11 expression construct, the full-length Kif11 (DDB_G0291039) sequence was amplified by PCR using genomic *D. discoideum* DNA as template. The Kif11 sequence was cloned into the *Bam*HI-site of a pDEX-based GFP expression vector [14]. Cells of the *D. discoideum* wild-type strain AX2, and Kif11-null cells (clone #12-3) [9] were cultivated in nutrient HL5 medium (Formedium) in polystyrene culture dishes at 21 °C.

For expression of GFP-Kif11, *D. discoideum* AX2 wild-type or Kif11-null cells were transformed with the GFP-Kif11 expression vector by electroporation using a Bio-Rad gene pulser at 0.8–0.9 kV and 3 μF in 4-mm cuvettes. Transformants were selected by addition of 10 μg blasticidin-S (ICN Biomedicals Inc.) or 10 μg geneticin (Sigma) per ml, and cloned on lawns of non-pathogenic *Klebsiella aerogenes* grown on SM-agar plates.

2.2. Immunofluorescence microscopy

GFP-Kif11-expressing and Kif11-null cells were allowed to settle on glass coverslips for 30 min and were fixed as described previously [15]. Briefly, after aspiration of the medium, cells were fixed in 50% PHEM buffer (30 mM PIPES, 12.5 mM HEPES, 5 mM EGTA, 1 mM $MgCl_2$, pH 6.9) freshly supplemented with 0.25% glutaraldehyde and 0.25% Triton X-100 for 5 min. Unreacted aldehyde groups were blocked with 1 mg/ml sodium borohydrate freshly dissolved in Soerensen phosphate buffer (14.6 mM KH_2PO_4, 2 mM Na_2HPO_4, pH 6.0) for 10 min. Fixed cells were immunostained with YL 1/2 antibody [16], or anti-Cenp68 antibody [17] to label α-tubulin or *Dictyostelium* centromeres, respectively. Primary antibodies were detected using goat anti-rat or goat anti-rabbit IgG coupled to either Alexa-568 or Alexa-488 dyes (Molecular Probes).

2.3. Imaging of living cells and fixed cell preparations

All imaging was performed using a LSM 510 Meta confocal microscope (Zeiss) equipped with 100 × or 63 × Neofluar 1.3 oil-immersion lenses. The excitation wavelengths and detection ranges used for imaging were 488 nm and 505–530 nm for GFP/Alexa488, and 543 nm and 585–615 nm for mRFP/Alexa568. Live-cell microscopy of *Dictyostelium* was carried out at 21 °C in open 35-mm μ-dishes (Ibidi). Before imaging, cells were washed twice in Soerensen phosphate buffer.

2.4. Expression and purification of Kif11

The full-length coding region of the *D. discoideum* gene *kif11* and the sequence encoding a C-terminally truncated Kif11 (Kif11ΔC comprising amino acids 1-486) were amplified by PCR using genomic DNA, and cloned into the expression vector pFastBac1 (Invitrogen). Kif11 and Kif11ΔC were expressed as C-terminally tagged GCN4-YFP-Flag-tag fusion proteins using the baculovirus expression system (Invitrogen) in Sf9 insect cells according to the manufacturers' instructions. Typically, 200 ml Sf9 cells were infected with baculoviruses at a concentration of 2×10^6 cells/ml, and Flag-tagged Kif11 constructs were purified as described by Brunnbauer et al. [18]. The protein was eluted with 200 μl elution

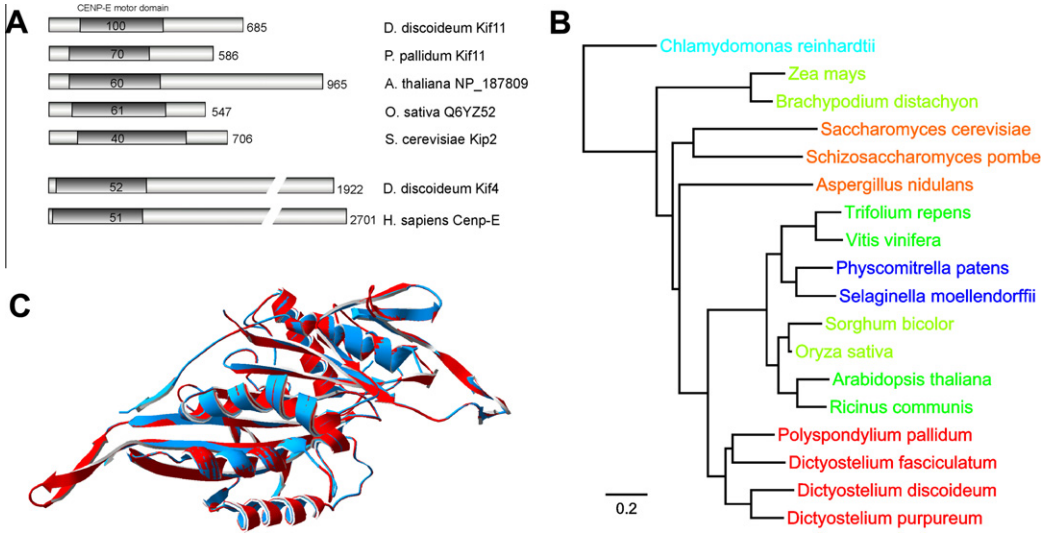

Fig. 1. Short CENP-E proteins in different organisms. (A) Schematic organization of short CENP-E proteins in comparison to the conventional kinetochore associated CENP-E proteins. The percentage of identity of the CENP-E domains of the different proteins is indicated in comparison to the *Dictyostelium* Kif11 CENP-E domain that was set to 100. (B) Phylogenetic tree of short variants containing a KISc_CENP-E domain that were identified by blast searches at NCBI. The tree was computed with the constraint-based multiple sequence alignment tool COBALT (fast minimal evolution) at NCBI [31]. The sequences used to compile the tree originate from diverse taxa, including eudicots (dark green; *Arabidospsis thaliana* (NP_565510), *Ricinus communis* (EEF32031), *Vitis vinifera* (XP_002265593), *Trifolium repens* (ADD09606)), monocots (light green; *Zea mays* (ACN33239), *Brachipodium distachyon* (XP_003577459), *Oryza sativa* (BAD17149), *Sorghum bicolor* (XP_002454643)), mosses (dark blue; *Physcomitrella patens* (XP_001764777), *Selaginella moellendorffi* (XP_002972220)), amoebozoa (red; *Dictyostelium discoideum* (EAL61950), *Dictyostelium purpureum* (DICPUDRAFT_96759), *Dictyostelium fasciculatum* (EGG21138), *Polyspondylium pallidum* (EFA85465)), ascomycetes (orange; *Aspergillus nidulans* (CBF74279), *Saccharomyces cerevisiae* (CAA78021), *Schizosaccharomyces pombe* (CAA22353)), and algae (light blue; *Chlamydomonas reinhardtii* (FLA10)). (C) Homology model of CENP-E domain of Kif11 (red) using 1T5C Chain A (blue; motor domain of human kinetochore protein CENP-E) as a template [32–34]. The sequence alignment of the CENP-E domains of *Dictyostelium discoideum* Kif11 and human CENP-E shares an overall identity of 51%. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

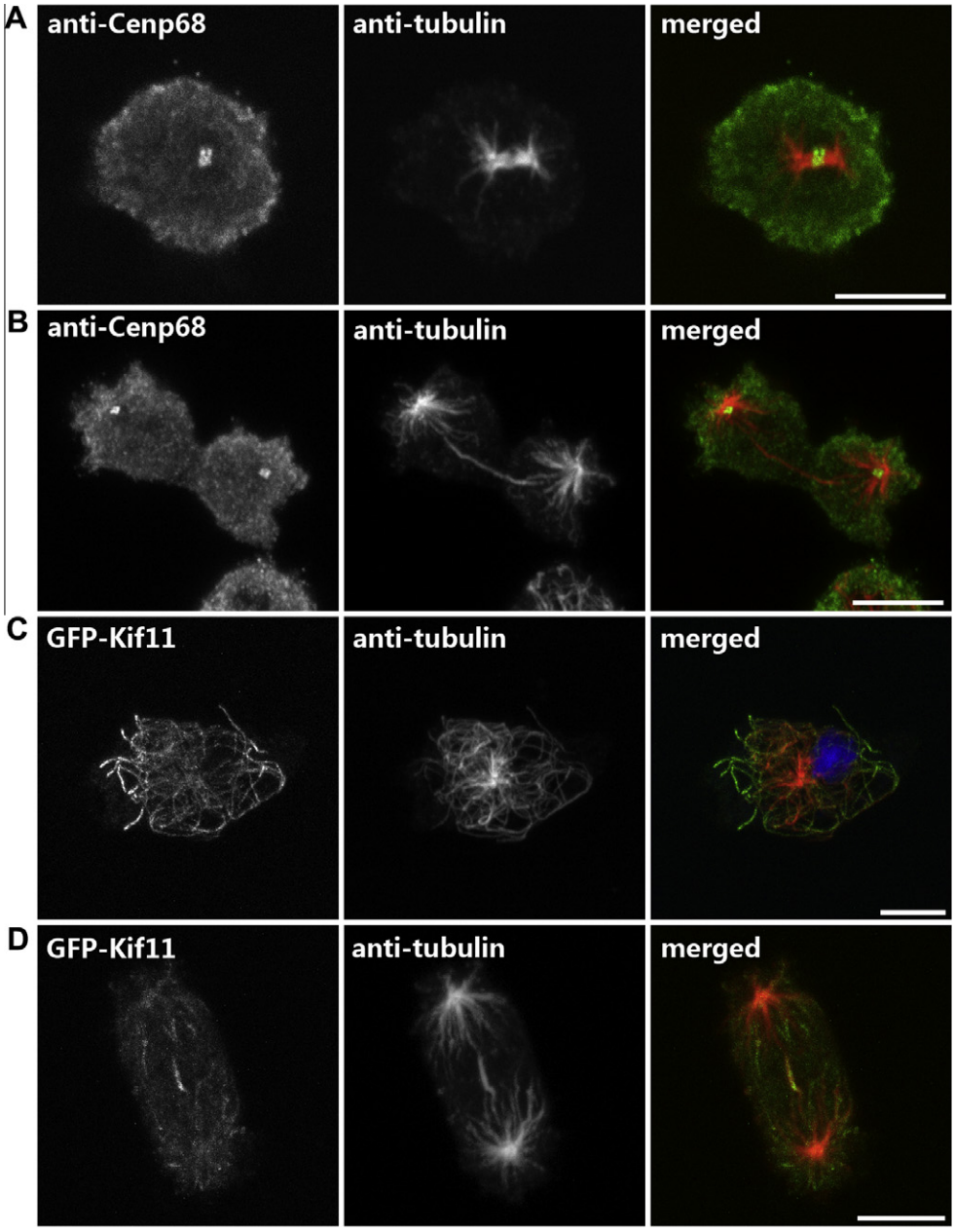

Fig. 2. Kinetochore dynamics in Kif11-null cells and localization of GFP-Kif11. (A and B) Depletion of Kif11 does not visually alter kinetochore structure. During metaphase, *Dictyostelium* kinetochores align between the spindle poles prior to separation by the spindle machinery in anaphase and telophase [35]. Kinetochores in Kif11-null cells were visualized using anti-Cenp68 antibodies, which label centromeres and kinetochores, throughout the entire cell cycle [17]. In Kif11-depleted cells, kinetochore structure and localization during metaphase (A) and telophase (B) appears identical to wild-type cells. Kif11-null cells were fixed with glutaraldehyde and stained with anti-Cenp68 (left) and anti-α-tubulin antibodies (center), respectively. In merged images (right), Cenp68 is shown in green and α-tubulin in red. (C and D) GFP-Kif11 displays predominant association with the + -TIP area of microtubules. Association of GFP-Kif11 to microtubules was found to be most pronounced near the microtubule plus ends in interphase cells (C), and the tips of spindle microtubules and the midbody region during late mitosis. (D) *Dictyostelium* wild-type cells expressing GFP-Kif11 (left) were fixed with glutaraldehyde, and microtubules were labeled using anti-α-tubulin antibody (center). In merged images (right), GFP-Kif11 is shown in green, α-tubulin in red, and DNA in blue. Bar = 5 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

buffer containing 1 × Flag peptide (Sigma) leading to protein yields ranging from 0.2 to 0.4 mg/ml.

2.5. ATPase assay

ATPase rates were calculated from the change in absorbance of NADH at 340 nm in the presence of an ATP regenerating system as described previously [18] with a final MgATP concentration of 2 mM. Microtubules were purified from porcine brain as described previously [19], and increasing amounts of taxol-stabilized (5 μM) microtubule filaments were added to the reaction mix (0–80 μM) to a final volume of 50 μl. Measurements were carried out in 96-well plates (Greiner) using a spectrophotometer (BioTek) at 23 °C. The data was analyzed with KaleidaGraph 3.6 software (Synergy Software) and fitted to the Michaelis–Menten kinetics [20].

2.6. Flow chamber preparation and motility assay

A flow chamber (volume 10 μl) was created with a glass slide/coverslip sandwich and sealed with silicone grease (GE Bayer).

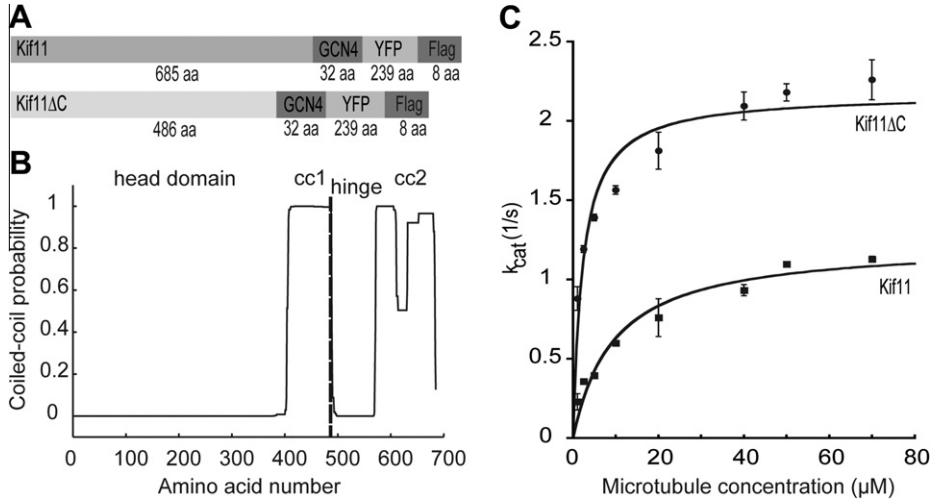

Fig. 3. Domain organization, coiled-coil prediction, and microtubule-stimulated ATPase activity of Kif11 and C-terminally truncated Kif11ΔC. (A) Domain structures of Kif11 and truncated Kif1ΔC. Linear maps show the generated constructs containing either the full-length motor protein Kif11 or its C-terminally truncated version Kif11ΔC, followed by a GCN4 motif to ensure dimerization, and a YFP for fluorescence detection. (B) Coiled-coil predictions for native and truncated motor proteins (based on COILS and [36]). The dashed line indicates position of the C-terminal truncation of the full-length protein. Head domain (amino acids 1-414), hinge region and coiled-coil (cc) domains are labeled. (C) Steady state ATPase activity, showing the plot of ATP-turnover against microtubule concentration fitted to Michaelis–Menten kinetics.

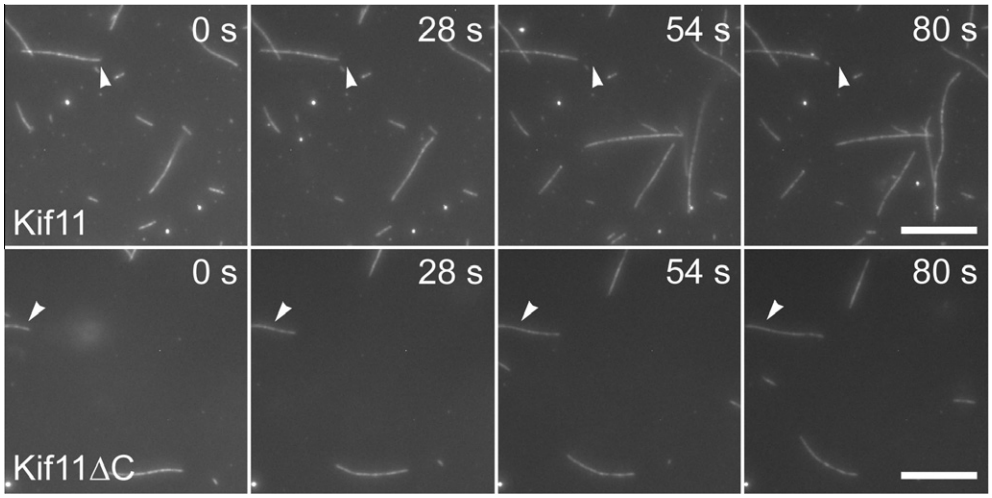

Fig. 4. Microtubule gliding assay of Kif11 and C-terminally truncated Kif11ΔC. Single frames of movies showing fluorescently labeled microtubules transported by multiple Kif11 (upper panel) and Kif11ΔC (lower panel) motors in motility assays. Fixed arrow heads indicate the movement of filaments. In comparison to Kif11, Kif11ΔC motors transport the filaments over a longer distance within the same time period, resulting in a higher gliding velocities (see Table 1). Scale bar = 10 μm.

Table 1
Steady-state ATPase values and motor gliding velocities of full-length and truncated Kif11 motors.

	Steady state ATPase		Motility
	K_{cat} (s^{-1})	K_M (μM)	V (nm/s)
Kif11	1.23 ± 0.09	9.6 ± 2.30	38 ± 7 (n = 38)
Kif11ΔC	2.17 ± 0.10	2.2 ± 0.52	90 ± 14 (n = 58)

Subsequently, the chamber was coated with 1 mg/ml BSA (Sigma), 1 mg/ml streptavidin (Sigma), and anti-Flag BioM2 antibodies (Sigma) respectively, with intervening washing steps using BRB buffer (80 mM Pipes, pH 6.9, 100 mM potassium acetate, 2 mM $MgCl_2$, 1 mM EGTA, 1 mM DTT) and 1 mg/ml BSA. Flow chambers were then perfused with 10 μl of motor protein solution (0.2–0.4 mg/ml) for 4 min. Unbound motors were removed with 34.5 μl of motility buffer containing fluorescent microtubules, labeled with Cy3 as previously described by Peloquin et al. [21] (BRB buffer, 2 mM ATP, 0.4% glucose, 0.145 mg/ml glucoseoxidase (Sigma, G2133), 0.0485 mg/ml catalase (Sigma, C3155), 0.2 mg/ml casein). Fluorescently labeled microtubules were recorded over time using an Axiovert 200 M microscope (Zeiss), and their velocities were measured using Axiovision software (Zeiss).

3. Results and discussion

3.1. Short CENP-E homologs in D. discoideum and other organisms

The *D. discoideum* genome encodes two kinesin-7 (CENP-E) family members, Kif4 and Kif11, containing an N-terminal CENP-E motor domain [1]. Generally, the mitotic CENP-E proteins are among the largest kinesins with long extended tails following the motor domain, and have been identified as essential kinetochore components involved in capture and stabilization of spindle microtubules. Genome analyses have also revealed short CENP-E

variants in plants, amoebae, and fungi, but their functions are largely unexplored.

Dictyostelium Kif4 (1922 amino acids) is a clear CENP-E ortholog with mitotic functions [9]. In contrast, Kif11 is the shortest of all the *Dictyostelium* kinesins (685 amino acids), and disruption of the *kif11* gene showed that Kif11 is not required for mitotic spindle assembly, cell growth or development [9], and thus seems to have functions distinct from Kif4. Closely related short CENP-E proteins are not only present in other amoebozoa but also in higher plants, fungi, mosses and algae (Fig. 1A and B). Structurally, the short CENP-E variants are characterized by an N-terminal region of approximately 100 amino acids that precedes the CENP-E motor domain: in the classical CENP-E proteins, this N-terminal region is much shorter (Fig. 1A). The C-terminal regions vary in length among the short CENP-E versions (150 to about 600 residues), but overall they are significantly shorter than the comparable region in the mitotic CENP-E proteins. Homology modeling using the structure of the motor domain of human CENP-E (1T5C.A; 2.5 angstrom) [13] as template revealed strong similarity of the folds that constitute the motor domain (Fig. 1C), indicating a close family realionship between the long and short versions.

3.2. Localization of GFP-Kif11 in D. discoideum

Classical CENP-E proteins have been shown localized to centromeric regions, and their loss causes severe changes in kinetochore morphology [22]. Knockout of Kif11 did not indicate an obvious function of this protein in mitosis. In order to investigate this in more detail, we labeled the kinetochores of dividing Kif11-null cells (Fig. 2A and B) using centromere-specific antibodies. In Kif11-null cells the kinetochore appearance is indistinguishable from wild-type cells, suggesting that Kif11 is not required for proper kinetochore assembly.

To analyze the localization of Kif11, we expressed a full-length GFP-tagged version of Kif11 in *Dictyostelium* cells. In interphase cells, GFP-Kif11 localizes along microtubules with a striking enrichment towards the microtubule plus ends as indicated by a higher fluorescence signal (Fig. 2C). The labeling of microtubules is also observed in live cells, where the signals appear to be enriched in clusters at the microtubule plus ends close to the cortex (Supplementary movie 1). During mitosis, Kif11 also colocalizes with microtubules, and is concentrated in the midzone and at microtubule ends contacting the distal cell poles (Fig. 2D). However, in contrast to the classical CENP-E isoforms, there does not appear to be any Kif11 labeling in the kinetochore region.

3.3. Functional characterization of D. discoideum Kif11

To investigate the mechanochemical properties of Kif11, we assayed full-length Kif11 and C-terminally truncated Kif11 (Kif11ΔC) polypeptides tagged with YFP (Fig. 3A). The motive to construct a truncated Kif11 was to test whether the C-terminus exerts a regulatory role on the activity of the motor (Fig. 3B). It has previously been shown that several kinesins are modulated by autoinhibition, due to action of a regulatory fold located in the central hinge region between the two coiled-coil domains [23–26]. The assumption is that this autoinhibition is relieved when the motor is converted to an extended conformation by binding to its cargo *in vivo* and *in vitro* [23,27–29]. Constitutively active motors have been generated either by introducing mutations in the hinge regions to prevent an autoinhibitory folding of the stalk, or by truncating the C-terminal tail regions of the motor [18,23,27]. Both methods prevent the interaction of the C-terminal tail regions with the catalytic head domains and ultimately results in unhibited ATPase activity of the motor.

The two *Dictyostelium* Kif11 motor constructs, full-length Kif11 and truncated Kif11ΔC (Fig. 3A) were expressed in insect cells and isolated using FLAG-tag affinity purification. To determine the velocities of the constructs, we employed *in vitro* gliding-filament assays using fluorescently labeled microtubules [18]. Surface-attached full-length Kif11 motors propelled the microtubules with a velocity of 38 nm/s, whereas the truncated motor protein Kif11ΔC displayed a velocity of 90 nm/s (Fig. 4, and Table 1). Both values are consistent with the mean velocities determined for the full-length mitotic kinesin CENP-E from *Xenopus* (30 ± 7.6 nm/s) [10].

To corroborate the results obtained from gliding assays, we performed microtubule-activated steady state ATPase assays. Consistent with the results described above, we observed a suppressed ATPase activity for the full-length construct Kif11 when compared to C-terminally truncated Kif11ΔC (Fig. 3C, and Table 1), suggesting a putative regulatory function of the tail inhibiting the enzymatically active head motor domains. The truncated mutant Kif11ΔC showed an ATP turnover of $k_{cat} = 2.2 \pm 0.1\ s^{-1}$, whereas the full-length Kif11 reached only $k_{cat} = 1.2 \pm 0.1\ s^{-1}$. Moreover, we observed an increased microtubule affinity for the truncated Kif11ΔC ($K_M = 2.2 \pm 0.5\ \mu M$) compared to the full-length Kif11 ($K_M = 9.6 \pm 2.3\ \mu M$).

The faster gliding rate and increased ATP turnover of Kif11Δ indicate that the suppression of the motor activity in wild type is relieved after removing the tail domain. For *Xenopus* CENP-E it was demonstrated recently that the motor activity is regulated by interaction between the tail and the motor domains [30]. In this case the autoinhibition of CENP-E was shown to be reversed by MPS1- or CDK1-cyclin B-mediated phosphorylation of the tail. These data together with results from our study suggest that regulatory mechanisms acting on the tail region can be responsible for modulation of the motor activity of Kif11.

4. Conclusion

Sequence analyses revealed that plants and fungi contain in addition to the mitotic kinesin CENP-E, a short CENP-E protein of so far unknown function. We have set out to explore the role and properties of the short non-mitotic CENP-E variant Kif11 in *Dictyostelium*. In summary, our results strongly suggest that Kif11 is a slow, plus-end directed transport motor with motor characteristics very similar to mitotic CENP-E isoforms. Our data also indicate that the motor activity is regulated by an unknown mechanism acting on the tail region and modulating the activity of the motor. However, in spite of the similarities, the short CENP-E variant Kif11 is involved in processes other than mitosis. Although not essential for cell viability, this subfamily of kinesin-7 motors is conserved in plants and fungi, and thus must serve some unique functions here that are not required in higher animal cells. Future work will concentrate on the nature of cargo interactions with this slow motor protein.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.01.030.

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